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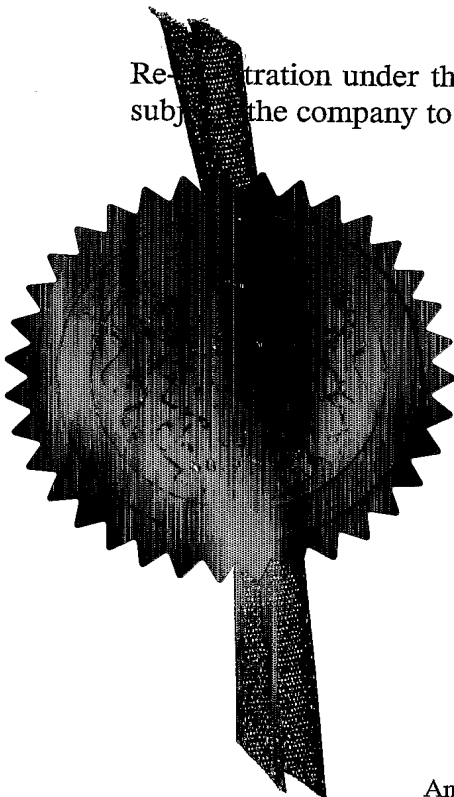
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P0177700 0.00-0317945.4

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2.	Patent application number (The Patent Office will fill in this part)	0317945.4		
3.	Full name, address and postcode of the or of each applicant (underline all surnames)	Gentronix Limited The Fairbairn Building 72 Sackville Street Manchester, M60 1QD		
	Patents ADP number (if you know it)			
	If the applicant is a corporate body, give the country/state of its incorporation	UNITED KINGDOM		
4.	Title of the invention	GENOTOXIC TESTING		
5.	Name of your agent (if you have one)	Marks & Clerk		
	"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)	43 Park Place Leeds LS1 2RY		
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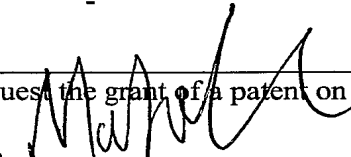
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## GENOTOXIC TESTING

The present invention relates to improved methods for detecting agents that cause or potentiate DNA damage and to molecules and transformed cells that may be usefully employed in such methods.

DNA damage is induced by a variety of agents such as ultraviolet light, X rays, free radicals, methylating agents and other mutagenic compounds. These agents may cause damage to the DNA that comprises the genetic code of an organism and cause mutations in genes. In microorganisms such mutations may lead to the evolution of new undesirable strains of the microorganism. For instance, antibiotic or herbicide resistant bacteria may arise. In animals these mutations can lead to carcinogenesis or may damage the gametes to give rise to congenital defects in offspring.

These DNA damaging agents may chemically modify the nucleotides that comprise DNA and may also break the phosphodiester bonds that link the nucleotides or disrupt association between bases (T-A or C-G). To counter the effect of these DNA damaging agents cells have evolved a number of mechanisms. For instance the SOS response in *E. coli* is a well characterised cellular response induced by DNA damage in which a series of proteins are expressed, including DNA repair enzymes, which repair the damaged DNA.

There are numerous circumstances when it is important to identify what agents may cause or potentiate DNA damage. It is particularly important to detect agents that cause DNA damage when assessing whether it is safe to expose a person to these agents. For instance a method of detecting these agents may be used as a mutagenesis assay for screening compounds that are candidate medicaments, food additives or cosmetics to assess whether or not the compound of interest induces DNA damage. Alternatively methods of detecting DNA damaging agents may be used to monitor for contamination of water supplies with pollutants that contain mutagenic compounds.

Various methods, such as the Ames Test, for determining the toxicity of an agent are known but are unsatisfactory for a number of reasons. For instance,

incubation of samples can take many days when it is often desirable to obtain genotoxic data in a shorter time frame. Furthermore, many known methods of detecting DNA damage (including the Ames Test and related methods) assay lasting DNA damage, as an endpoint, either in the form of misrepaired DNA (mutations and recombinations) or unrepaired damage in the form of fragmented DNA. However most DNA damage is repaired before such an endpoint can be measured and lasting DNA damage only occurs if the conditions are so severe that the repair mechanisms have been saturated.

An improved genotoxic test is disclosed in WO 98/44149. This specification concerns recombinant DNA molecules comprising a regulatory element that activates gene expression in response to DNA damage operatively linked to a DNA sequence that encodes a light emitting reporter protein. Such DNA molecules may be used to transform a cell and such cells used in a genotoxic test for detecting for the presence of an agent that causes or potentiates DNA damage. The cells may be subjected to an agent and the expression of the light emitting reporter protein from the cell indicates that the agents cause DNA damage.

The genotoxic tests described in WO 98/44149 detect the induction of repair activity that prevents an endpoint being reached. The method described in WO 98/44149 may therefore be used to detect for the presence of DNA damaging agents.

WO 98/44149 describes a number of useful genetic constructs that may be used to transform a cell such that it may be used in a genotoxic test. One such construct is yEGFP-444 (illustrated in Figure 12 of WO 98/44149) and the present invention is based upon a novel type of construct that was identified during developmental work carried out with yEGFP-444.

According to a first aspect of the invention, there is provided a recombinant vector comprising a recombinant DNA molecule comprising a regulatory element that activates gene expression in response to DNA damage operatively linked to a DNA sequence that encodes a light emitting reporter protein and a DNA vector characterised in that the vector comprises an origin of replication; at least one

selectable maker; and when used to transform a cell, does not alter the sensitivity of the cell to geneticin.

According to a second aspect of the invention, there is provided a cell containing a recombinant vector in accordance with the first aspect of the present invention.

According to a third aspect of the present invention, there is provided a method of detecting for the presence of an agent that causes or potentiates DNA damage comprising subjecting a cell in accordance with the second aspect of the present invention to an agent and monitoring the expression of the light emitting reporter protein from the cell.

By "regulatory element" we mean a DNA sequence that regulates the transcription of a gene with which it is associated.

By "operatively linked" we mean that the regulatory element is able to induce the expression of the reporter protein.

By "reporter protein" we mean a protein which when expressed in response to the regulatory element of the DNA molecule of the invention is detectable by means of a suitable assay procedure.

By "pWDH445" we mean the expression vector illustrated in Figure 5 of this specification. pWDH445 was originally called yEGFP444 and was originally disclosed in Figure 12 of WO 97/44149.

The method of the third aspect of the invention represents a novel cost-effective genotoxicity screen, that may be used to provide a pre-regulatory screening assay for use by the pharmaceutical industry and in other applications where significant numbers of compounds need to be tested. It provides a higher throughput and a lower compound consumption than existing eukaryotic genotoxicity assays and is sensitive to a broad spectrum of mutagens and, importantly, clastogens.

The method of the third aspect of the invention is suitable for assessing whether or not an agent may cause DNA damage. It is particularly useful for detecting agents that cause DNA damage when assessing whether it is safe to expose a person to DNA damaging agents. For instance, the method may be used as a mutagenesis assay for screening whether or not known agents, such as candidate medicaments, foodstuffs or cosmetics, induce DNA damage. Alternatively the method of the invention may be used to monitor for contamination of water supplies with pollutants containing DNA damaging agents.

The method of the third aspect of the invention may equally be used for assessing whether an agent may potentiate DNA damage. For example, certain agents can cause accumulation of DNA damage by inhibiting DNA repair (for instance by preventing expression or function of a repair protein) without directly inflicting DNA damage. These agents are often known as co-mutagens.

The present invention is based upon work conducted on the vectors disclosed in WO 98/44149. The inventors have found that a spontaneous rearrangement of the known vector pWDH445 occurred that resulted in a brighter reporter protein. They therefore conducted experiments, which are described in more detail in the Example, to characterise this fortuitous mutation and to isolate recombinant vectors according to the first aspect of the present invention.

The vector pWDH445 is illustrated in figure 5. The inventors have found that, surprisingly, deletion of the *kanMX3* gene function (i.e. removing geneticin resistance) from the pWDH445 gene results in a vector capable of expressing the reporter protein (GFP in the case of pWDH445) such that the signal from the report is significantly greater when DNA damage occurs.

Preferred vectors according to the first aspect of the invention comprise pWDH445 (y EGFP-444 in WO 98/44149) with disruption of the *kanMX3* gene.

The *kanMX3* gene may be disrupted such that a mutation occurs by means of deletion, substitution or addition of nucleotides provided that genetecin resistance is



impaired. A most preferred recombinant vector according to the first aspect of the invention is pGen001. The nucleotide sequence of pGen001 is given in figures 16 and 17.

It will be appreciated that the recombinant DNA molecule in pGen001 is interchangeable. Accordingly the RAD54 promoter – y EGFP expression cassette depicted in figure 16 may be easily replaced (e.g. by Bam H1 / Ascl digestion) to insert another recombinant DNA molecule according to the invention (see below).

The regulatory element of the recombinant DNA molecule activates expression of the reporter protein when DNA damage occurs. Such regulatory elements ideally comprise a promoter sequence which induces RNA polymerase to bind to the DNA molecule and start transcribing the DNA encoding the reporter protein. The regulatory element may also comprise other functional DNA sequences such as translation initiation sequences for ribosome binding or DNA sequences that bind transcription factors which promote gene expression following DNA damage. Regulatory elements may even code for proteins which act to dislodge inhibitors of transcription from the regulated gene and thereby increase transcription of that gene.

Preferred regulatory elements are DNA sequences that are associated in nature with the regulation of the expression of DNA repair proteins. For instance, the regulatory elements from genes such as RAD2, RAD6, RAD7, RAD18, RAD23, RAD51, RAD54, CDC7, CDC8, CDC9, MAG1, PHR1, DIN1, DDR48 and UB14 from yeast may be used to make recombinant DNA molecules according to the first aspect of the invention.

A preferred regulatory element comprises the promoter and 5' regulatory sequences of the RAD54 repair gene. Such a regulatory element may be derived from yeast and particularly *Saccharomyces cerevisiae*. It is most preferred that the regulatory element comprises the promoter and 5' regulatory sequences of the RAD54 repair gene which correspond to the DNA sequence identified in WO 98/44149 or a functional analogue or fragment thereof.

The DNA sequences that encode a light emitting reporter protein may code for

any light emitting protein, however it is preferred that the DNA sequences code for a protein that is fluorescent.

Preferred DNA sequences that encode a light emitting reporter protein code for Green Fluorescent Protein (GFP) and light emitting derivatives thereof. GFP is from the jelly fish *Aequorea Victoria* and is able to absorb blue light and re-emits an easily detectable green light and is thus suitable as a reporter protein. GFP may be advantageously used as a reporter protein because its measurement is simple and reagent free and the protein is non-toxic.

Derivatives of GFP include DNA sequences encoding for polypeptide analogues or polypeptide fragments of GFP which are able to emit light. Many of these derivatives absorb and re-emit light at wavelengths different to GFP found endogenously in *Aequorea victoria*. For instance, preferred DNA molecules according to the first aspect of the invention have a DNA sequence that encodes the S65T derivative of GFP (in which serine 65 of GFP is replaced by a threonine). S65T GFP has the advantage that it is brighter than wild-type GFP (when excited at its longest-wavelength peak) and shows only slow photobleaching. Furthermore S65T GFP produces a good quantum yield of fluorescence and matches the output of argon ion lasers used in fluorescence activated cell sorters. Cells according to the second aspect of the invention which contain DNA molecules coding S65T GFP may be used according to the method of the fourth aspect of the invention and are particularly useful when light emission is measured from cell extracts.

A most preferred DNA sequence encodes for a yeast enhanced GFP (yEGFP) such as the GFP derivative described by Cormack et al. (1997) (in *Microbiology* 143 p303-311). Such yEGFP has an amino acid sequence which is biased for usage in yeast. Thus yEGFP is particularly suitable for transforming cells according to the second aspect of the invention which are yeast. Furthermore we have found that light emitted from yEGFP in such yeast is even greater than that emitted by S65T derivatives.

Recombinant DNA molecules coding yEGFP are also useful because yEGFP is less heat sensitive than nascent GFP.

Most preferred recombinant DNA molecules comprise a RAD54 regulatory element operatively linked to a DNA sequence that encodes a GFP or light emitting derivative thereof.

The recombinant vector according to the first aspect of the present invention may for example be a plasmid, cosmid or phage. Such recombinant vectors are of great utility when replicating the DNA molecule. Furthermore recombinant vectors are highly useful for transforming cells with the DNA molecule and may also promote expression of the reporter protein.

Recombinant vectors may be designed such that the vector will autonomously replicate in the cytosol of the cell. In this case, elements which induce DNA replication may be required in the recombinant vector. A suitable element is derived from the 2 $\mu$  plasmid. Such replicating vectors can give rise to multiple copies of the DNA molecule in a transformant and are therefore useful when over-expression (and thereby increased light emission) of the reporter protein is required.

Preferably recombinant vectors may be formed from PFA vectors or derivatives thereof which are known to the art (see Wach et al. (1994) Yeast 10 p1793-1808).

Preferred recombinant vectors are derived from yEGFP-444 disclosed in WO 97/44149 (the disclosure of which is incorporated herein by reference). yEGFP-444 is also known as pWDH445.

According to the second aspect of the invention the recombinant vector is incorporated within a cell. Such host cells may be prokaryotic or eukaryotic. Suitable host cells include bacteria, plant, yeasts, insect and mammalian cells. Preferred host cells are yeast cells such as *Saccharomyces cerevisiae*. Yeast are preferred because they can be easily manipulated like bacteria but are eukaryotic and therefore have DNA repair systems that are more closely related to humans than those of bacteria.

Another benefit of using yeast cells as a host is that DNA repair systems are inducible in yeast unlike in humans where the repair systems are largely constitutive.

Preferred yeast cells include:

- (i) Y485 in haploid form;
- (ii) Y486 (also known as FF18984) in haploid form;
- (iii) Y485/486 in diploid form;
- (iv) FY73
- (v) YLR030w.alpha.; and
- (vi) Y300.

These strains may all be found in national yeast strain collections.

We have found that (i), (ii) and (iii) above are particularly useful strains for use according to the method of the invention.

Host cells used for expression of the protein encoded by the DNA molecule are ideally stably transformed, although the use of unstably transformed (transient) cells is not precluded.

Transformed cells according to the second aspect of the invention may be formed by following procedures described in the Example. The cell is ideally a yeast cell (for instance one of the strains described above). Such transformed cells may be used according to the method of the third aspect of the invention to assess whether or not agents induce or potentiate DNA damage. GFP expression is induced in response to DNA damage and the light emitted by GFP may be easily measured using a fluorimeter as an index of the DNA damage caused. For instance, the light emitted by GFP at 511 nm (after excitation between 475 and 495 nm--e.g. 488 nm) in response to DNA damage, may be evaluated either in a suspension of a defined number of whole cells or from a defined amount of material released from cells following breakage. Alternatively light emitted by GFP at 520 nm may be evaluated through a 535nm filter.

The method of the third aspect of the invention is particularly useful for detecting agents that induce DNA damage at low concentrations. The methods may be used to screen compounds, such as candidate medicaments, food additives or cosmetics, to assess whether it is safe to expose a living organism, particularly people, to such compounds.

Alternatively the method of the third aspect of the invention may be employed to detect whether or not water supplies are contaminated by DNA damaging agents or agents that potentiate DNA damage. For instance, the methods may be used to monitor industrial effluents for the presence of pollutants that may lead to increased DNA damage in people or other organisms exposed to the pollution.

When the methods are used to detect whether or not water supplies are contaminated, the cells according to the second aspect of the invention are ideally unicellular organisms such as bacteria, algae, protoza and particularly yeast.

The expression of light emitting reporter protein may be monitored according to the method of the invention from cell extracts or from samples containing intact, whole cells.

There are several advantages associated with the use of whole cells. As there is no requirement to break open cells, the number of treatment steps is reduced. The production of extracts requires cell-harvesting, washing, breakage with glass beads and centrifugation to clear the extract. The reduction in treatment steps also reduces the risk of errors arising in handling and makes the method much faster. Furthermore cell density and light emission can be made simultaneously giving greater sensitivity. Therefore the method of the invention is preferably performed by growing cells transformed with a recombinant vector according to the first aspect of the invention (such as pGEN001), incubating the cells with the agent which putatively causes DNA damage for a predetermined time and monitoring the expression of the light emitting reporter protein directly from a sample of the cells.

When whole cells are used they are preferably contained in low fluorescence growth medium. This can obviate the need to wash the cells before measurements are made and therefore reduce the number of steps in the method further. For instance, preferred yeast according to the third aspect of the invention may be grown in F1 medium (described in Walmsley et al. (1983) Mol. Gen. Genet. 192 p361-365 and the Example).

According to a preferred embodiment of the method of the invention FF18984 cells may be transformed with pGEN001 and grown in F1 medium. A putative DNA damaging agent (e.g. a food additive or potential medicament or an agent contained within a water sample or effluent sample) may then be added to the F1 medium containing the cells. The cells are then allowed to grow for a defined period of time after which a sample of the cells is removed and fluorescence measured therefrom. This measurement may be effected by estimating the cell concentration and fluorescence in the sample using nephelometry (light scattering). For example, cells can be illuminated at 600 nm and the scattered light (at 600 nm) estimated at 90 degrees to the incident beam. The light emitted by GFP can be measured by excitation at 475-495 nm (e.g. 487 nm) and fluorescent light emitted at 518 nm measured at 90 degrees to the incident beam. Both measurements may be made in a single cuvette. A normalised GFP light emission is calculated by dividing the GFP fluorescence value by the whole cell light scattering value (at 600 nm). This embodiment of the invention has the advantage that it may be easily carried out with a minimum of steps (i.e an incubation period followed by direct fluorescence measurement).

The method of the invention should ideally employ sensitive fluorimeters and reduce light scattering in order that light emission can be accurately measured from the reporter protein. We have found that sensitivity can be improved by using a 487 nm filter which is introduced between the sample chamber and the emission-detector of the fluorimeter. Such a filter further reduces the impact of light scattering and improves the sensitivity of the method when samples containing whole cells are used.

Preferred methods of fluorescence detection and quantitation are given in 1.2.5.5 of Example 1, 2 and 3.

A preferred method of fluorescence detection is described in US 6, 509, 161. This method is particularly useful when the light emitting report is GFP or a derivative thereof.

A preferred method of testing for DNA damage according to the third aspect of the invention comprises the steps of : (1) preparing a microplate for use in an assay; (2) conducting the assay in the microplates; (3) collecting and analysing the data; and (4) making a judgment on DNA damage and the consequences.

Details of steps (1) – (4) for conducting a most preferred test for DNA damage are given below.

(1) Microplate preparation.

Assays were carried out in 96 well, black, clear-bottomed microplates. For example Matrix ScreenMates, Cat. No. 4929, Apogent Discoveries, USA, or Corning (BV, Netherlands: Cat. No. 3651). A number of alternative microplates were assessed, though the variable background absorbance and fluorescence both within and between plates from individual manufacturers were generally unacceptable, leading to the currently preferred choice. It will therefore be appreciated that microplates used according to the invention preferably have consistent absorbance and fluorescence between plates and batches thereof.

The assay plates can be filled using a liquid handling robot. For example the MicroLabS single probe, from Hamilton GB Ltd., Birmingham or a Genesis 8-probe robot (Tecan UK Ltd. Theale. UK). Microplates can also be filled rapidly and effectively using a multi-channel pipette.

(2) Assay

The following standard protocol may be followed. A 1 mM stock of a test chemical, or sample containing an agent that putatively caused DNA damage, was prepared in 2% v/v aqueous DMSO, and used to make 2 identical dilution series across a 96 well microplate and a 'control' (see below). To achieve this, 150 microlitres of the test chemical solution were put into 2 microplate wells. Each sample was serially diluted by transferring 75 microlitres into 75 microlitres of 4%

DMSO, mixing, and then taking 75 microlitres out and into the next well. This produced 9 serial dilutions of 75 microlitres each.

Controls were added as follows:

- a. Test compound/ sample containing agent alone, to provide information on compound absorbance/fluorescence
- b. Yeast cultures diluted with 4% DMSO alone, to give a measure of maximum proliferative potential
- c. MMS as a genotoxicity control: 'high' = 0.00125% v/v, 'low' = 0.0001875% v/v
- d. Methanol as a cytotoxicity control: 'high' = 3.5% v/v, 'low' = 1.5% v/v.
- e. Growth medium alone, to confirm sterility/lack of contamination

Stationary phase cultures of yeast cells according to the invention (e.g. GenT01 and GenC01) were diluted to an optical density (OD<sub>600nm</sub>) = 0.2 in double strength F1 medium (Billinton et al., 1998). 75 microlitres of the yeast suspension were added to each well of the diluted chemical: GenT01 to one series and GenC01 to the second, and to appropriate standards and controls. After the plates were filled, they were sealed using either a gas permeable membrane (for example Breath-easy, Diversified Biotech, USA) or a plastic lid, and then incubated without shaking, overnight at 25°C.

### (3) Data collection and handling.

Following overnight incubation, fluorescence and absorbance data were collected from the microplates. Two different microplate readers which combine fluorescence and absorbance functionality have been used, and comparable data were obtained. These were a Tecan Ultra-384 (Tecan UK Ltd.): excitation 485 nm / emission 535 nm with an additional dichroic mirror (reflectance 320 nm – 500 nm, transmission 520 nm – 800 nm), and a BMG PolarStar (BMG Labtechnologies, Germany): excitation 485 nm / emission 520 nm. Absorbance was measured through a 620 nm filter in both instruments. The data were transported into a Microsoft Excel spreadsheet, and converted to graphical data (see typical data in Figs 18 and 19 for Example 2). Data processing is minimal: absorbance data give an indication of



reduction in proliferative potential and these data were normalised to the untreated control (=100% growth). Fluorescence data were divided by absorbance data to give 'brightness units', the measure of average GFP induction per cell. These data were normalised to the untreated control (=1). In this way, one can distinguish between a small number of highly fluorescent cells and a large number of weakly fluorescent cells. In order to correct for induced cellular autofluorescence and intrinsic compound fluorescence, the brightness values for the GenC01 strain were subtracted from those of GenT01. This makes visual assessment of the data more reliable. All the data were checked with and without this correction, and the decision (see below), on whether or not a compound was classified as being genotoxic, was not affected.

#### (4) Decision thresholds.

It is useful to have clear definitions of positive and negative results from routine assays and such definitions have been derived, taking into account the maximum noise in the system and data from chemicals where there is a clear consensus on genotoxicity and mechanism of action. Naturally it is also possible for users to inspect the numerical and graphical data and draw their own conclusions. For example an upward trend in genotoxicity data that did not cross the threshold might still distinguish two compounds. The decision thresholds were set as follows:

The cytotoxicity threshold is set at 80 % of the cell density reached by the untreated control cells. This is greater than 3 times the standard deviation of the background. A positive cytotoxicity result (+) is concluded if 1 or 2 compound dilutions produce a final cell density lower than the 80% threshold. A strong positive cytotoxicity result positive (++) is concluded when either (i) three or more compound dilutions produce a final cell density lower than the 80% threshold or (ii) at least one compound dilution produces a final cell density lower than a 50% threshold. A negative result (-) is concluded when no compound dilutions produce a final cell density lower than the 80% threshold. The lowest effective concentration (LEC) is the lowest test compound concentration that produces a final cell density below the 80% threshold.

The compound absorbance control allows a warning to be generated if a test compound is significantly absorbing. If the ratio of the absorbance of the compound

control well to a well filled with diluent alone is  $> 2$ , there is a risk of interference with interpretation. The cytotoxicity controls indicate that the yeast is behaving normally. The 'high' methanol standard should reduce the final cell density to below the 80% threshold, and should be a lower value than the 'low' standard.

The genotoxic threshold is set at a relative GFP induction of 1.3 (i.e. a 30% increase). This is greater than 3 times the standard deviation of the background. A positive genotoxicity result (+) is concluded if 1 or 2 compound dilutions produce a relative GFP induction greater than the 1.3 threshold. A strong positive genotoxicity result (++) is concluded if either (i) three or more compound dilutions produce a relative GFP induction greater than the 1.3 threshold or (ii) at least one compound dilution produces a relative GFP induction greater than a 1.6 threshold. A negative genotoxicity result (-) is concluded where no compound dilutions produce a relative GFP induction greater than the 1.3 threshold. The LEC is the lowest test compound concentration that produces a relative GFP induction greater than the 1.3 threshold. The genotoxic controls demonstrate that the strains are responding normally to DNA damage. The 'high' MMS standard must produce a fluorescence induction  $> 2$ , and be a greater value than the 'low' MMS standard. Anomalous brightness data is generated when the toxicity leads to a final cell density less than 30% that of the blank. Genotoxicity data is not calculated above this toxicity threshold. Compounds that tested negative for genotoxicity, were re-tested up to 10mM, or to the limit of solubility or cytotoxicity.

The compound fluorescence control allows a warning to be generated when a compound is highly auto-fluorescent. If the ratio of the fluorescence of the compound control well to a diluent filled well is  $> 5$ , there is a risk of interference with interpretation. In these cases (four in this study), fluorescence polarisation can be used to distinguish GFP from other sources of fluorescence (Knight *et al.*, 2000, 2002). Both the Tecan and BMG instruments have this facility. Occasionally, compounds though not fluorescent themselves, induce cellular auto-fluorescence. This is apparent from rising brightness in the control (GenC01) strain in the absence of fluorescence from the chemical-only control. The routine subtraction of GenC01 from GenT01 data removes this interference from the data.

The present invention will now be described, by way of examples, with reference to the accompanying drawings in which:

**Figure 1** shows the difference between the brightness values obtained from FF18984 cells transformed with pWDH445, exhibiting normal levels of brightness (■) and enhanced brightness (■) without and with exposure to MMS. U, untransformed FF18984 cells not treated with MMS; U MMS, untransformed cells exposed to 0.005% MMS (15 hours); T, cells transformed with pWDH445 but not treated with MMS; T MMS, cells transformed with pWDH445 and exposed to 0.005% MMS; Brightness, fluorescence intensity (F int) corrected for culture density using the intensity of scattered light at 600 nm (Neph600). Each bar represents the average brightness value of three independent cultures. Both uninduced and MMS-induced brightness values are clearly significantly enhanced in the new transformants relative to the original transformants.

**Figure 2** shows an ethidium bromide stained agarose gel of pWDH445 cut with *Bam*H I and *Asc* I. The lanes contain the following: Lane 1, pWDH445; Lane 2, plasmid isolated from an enhanced brightness transformant; Lane 3, pWDH445 isolated from a normal brightness yeast transformant; and Lane 4, a second isolate from a transformant exhibiting enhanced brightness. *Bam*H I-*Asc* I restriction of pWDH445 liberates a fragment of 3.2 kb (boxed) bearing *HO-RAD54* promoter-*yEGFP* cassette. A band representing a 3.2 kb-sized fragment is visible in all four lanes.

**Figure 3** shows FF18984 cells transformed with pWDH445 and cells re-transformed with re-isolated pWDH445 or rearranged plasmid (two independent isolates) isolated from transformants of enhanced brightness. The four sets of transformants were replica-plated onto SD medium deficient in uracil and YPD medium incorporating 200  $\mu\text{gml}^{-1}$  geneticin (G418) and incubated at 30°C for 3 days. All four sets of transformants grew on SD-ura but only the cells bearing pWDH445 and re-isolated pWDH445 grew on YPD+G418.

**Figure 4** demonstrates that FF18984 cells re-transformed with rearranged plasmid (■) are brighter than FF18984 cells bearing pWDH445 (■) without and with exposure to 0.005% MMS. U, untransformed FF18984 cells not exposed to MMS; U MMS, untransformed cells exposed to 0.005% MMS (15 hours); T, cells transformed with pWDH445 or rearranged plasmid not exposed to MMS; T MMS, cells transformed with pWDH445 or rearranged plasmid and exposed to 0.005% MMS; Brightness, fluorescence intensity (F int) corrected for culture density using the intensity of scattered light at 600 nm (Neph600). Each bar represents the average brightness value of six independent cultures. Cells re-transformed with the re-isolated rearranged plasmid are still brighter than normal pWDH445 transformants both without and with exposure to MMS.

**Figure 5** shows the restriction map of pWDH445 created for and used in this study. Base-pair sequences of the plasmid constituents were analysed for restriction enzyme cleavage sites using software available on the Stanford Genome Database (SGD) web-site. Major plasmid components are shown as blocked sections with the plasmid backbone (mainly bacterial sequences, such as the origin of replication) drawn

represented by a line (—). The *RAD54* promoter (■) -*yEGFP* (■) cassette and the *kanMX3* cassette (■) are shaded to highlight the main regions of interest, whilst *Amp<sup>r</sup>* and *URA3* are represented by open block arrows. The arrows represent the direction of transcription for individual components.

**Figure 6** shows an ethidium bromide stained agarose gel of the four plasmids (pWDH445, re-isolated pWDH445, and two rearranged plasmids) digested with *Xba* I. The lanes were loaded as follows: Lane 1, pWDH445; Lane 2, rearranged plasmid; Lane 3, re-isolated pWDH445; Lane 4, second rearranged plasmid; Lane 5, 500 bp ladder (5-0.5 kb) marker DNA. *Xba* I restriction of both rearranged plasmids produced a novel band representing a fragment of ~4.4 kb, which was not detected for pWDH445 and re-isolated pWDH445. The rearranged plasmids also appear to be missing one band of a 6 kb doublet observed for the pWDH445 digestions. Extra bands in lane 2 reveal that one rearranged plasmid is larger than pWDH445, while the loss of a band from the doublet (and no extra bands) suggest that the rearranged plasmid loaded in lane 4 is smaller than pWDH445.

**Figure 7** shows an ethidium bromide stained agarose gel of the four plasmids (pWDH445, re-isolated pWDH445, and two rearranged plasmids) restriction digested with *Sca* I. The lanes were loaded as follows: Lane 1, 500 bp ladder (5-0.5 kb) molecular weight marker DNA; Lane 2, pWDH445; Lane 3, larger rearranged plasmid; Lane 4, re-isolated pWDH445; Lane 5, smaller rearranged plasmid. Bands representing fragments sized ~1.8 kb and ~4.1 kb are present in all four lanes (lanes 2-5) but the band representing a fragment of 1.3 kb (present for pWDH445 and re-isolated pWDH445) is not exhibited in the digestion of either rearranged plasmid (lanes 3 and 5).

**Figure 8** presents an ethidium bromide stained agarose gel of the four plasmids (pWDH445, re-isolated pWDH445, and two rearranged plasmids) restriction digested with *Pst* I. Lanes were loaded as follows: Lane 1, 500 bp ladder molecular weight marker DNA; Lane 2, pWDH445; Lane 3, larger rearranged plasmid; Lane 4, re-isolated pWDH445; Lane 5, smaller rearranged plasmid. Lanes 2 and 4 produced identical banding patterns whilst those in lanes 3 and 5 (rearranged plasmids) are significantly different. Only lanes 2 and 4 exhibit a band representing a fragment of ~2.5 kb. Lanes 2 – 5 all exhibit bands representing fragments of ~1.6, ~1.4, and ~1.3 kb, but lanes 3 and 5 also showed an extra band representing a fragment of 1.5 kb.

**Figure 9** shows an ethidium bromide stained agarose gel of *Pvu* I-digested pWDH445, re-isolated pWDH445, and rearranged plasmids. Lanes loaded as follows: Lane 1, pWDH445; Lane 2, larger rearranged plasmid; Lane 3, re-isolated pWDH445; Lane 4, smaller rearranged plasmid; Lane 5, 500 bp ladder molecular weight marker DNA. Lanes 1 and 3 exhibited the expected banding pattern, whereas neither rearranged (lanes 2 and 4) plasmid exhibits bands representing fragments of 1.2 and 0.7 kb. The larger rearranged plasmid (lane 2) also shows two novel bands representing fragments of ~3.5 kb and 1 kb.

**Figure 10** shows an ethidium bromide stained agarose gel of the four plasmids (pWDH445, re-isolated pWDH445, and two rearranged plasmids) digested with *Sac* I. Lanes were loaded with the following digested plasmids: Lane 1, pWDH445; Lane 2, larger rearranged plasmid; Lane 3, re-isolated pWDH445; Lane 4, smaller rearranged

plasmid; Lane 5, 500 bp ladder molecular weight marker DNA. *Sac* I liberates a 2.1 kb *kanMX* internal fragment from pWDH445 and both lanes 1 and 3 exhibit a band representing a 2.1 kb fragment. Neither rearranged plasmid (lanes 2 and 4) reveals such a band after *Sac* I restriction.

**Figure 11** presents an ethidium bromide stained agarose gel of *Xba* I-digested pWDH445 and pWDH445 with *kanMX* enzymically removed. Lanes were loaded as follows: Lanes 1 and 13, pWDH445; Lanes 2-6, 8, 9, 11, and 12, plasmid isolated from ampicillin-resistant, kanamycin-sensitive *E. coli* transformants; Lane 10, plasmid isolated from an ampicillin-resistant, kanamycin-resistant *E. coli* transformant; Lane 7, 500 bp ladder molecular weight marker DNA. Lanes 1 and 13 exhibit a doublet band representing two fragments of ~6 kb, which is also seen for the plasmid from the kanamycin-resistant colony (lane 10). All other lanes containing digested plasmid show a single band representing a fragment of ~6 kb and a second band representing a fragment of ~4.5 kb.

**Figure 12** represents the brightness values of FF18984 cells transformed with modified pWDH445 (enzymically removed *kanMX*) (■) compared with those from cells transformed with pWDH445 (■). U, untransformed FF18984 cells not exposed to MMS; U MMS, untransformed cells exposed to 0.005% MMS (15 hours); T, FF18984 cells transformed with pWDH445 or modified pWDH445 but not exposed to MMS; T MMS, cells transformed with pWDH445 or modified pWDH445 exposed to 0.005% MMS; Brightness, fluorescence intensity (F int) corrected for culture density using the intensity of scattered light at 600 nm (Neph600). Each bar represents the average brightness value of ten independent cultures. Cells bearing the modified pWDH445 exhibit enhanced brightness comparable to that of cells re-transformed with isolated rearranged plasmid.

**Figure 13** shows a schematic of the flow-through fluorescence detector with a blown-up schematic of the detector orientations within the light-tight box, and a further blown-up cartoon of the excitation beam and emissions, relative to the flow cell. The top section shows the overall layout of the instrument, beginning with cultures incubating in a water-bath shaker. Alternatively, samples can be injected *via* the sample injection valve. Culture/sample is pumped peristaltically into the light-tight instrument enclosure (grey box), wherein excitation occurs and emission is detected. The source of the excitation beam is an argon-ion laser (■). Within the instrument enclosure (see panel blown-up from the grey box) is a duplicated detector set-up. A beam-splitting cube send excitation light (488 nm) to both sets of detectors, each of which consists of an optical flow cell, photomultiplier tube (PMT), silicon photodiode (SPD), and optical filters (see key panel for colour-coding). Fluorescence is detected by the PMT whilst scattered light (as a measure of cell or particulate density) is detected by the SPD, both of which are situated perpendicular to the direction of the excitation beam. Orientation of the excitation beam and the emission detection is simplified in the bottom panel.

**Figure 14** depicts the detection of fluorescence polarisation with the flow-through fluorescence detector. Sample circulating through the flow cell is excited by plane-polarised laser light (vertical arrows represent the plane of polarisation) and scattered light is detected by an SPD placed at the other side of the flow cell in line with the beam of excitation. Emitted fluorescence is less polarised than the excitation light

and this is represented by vertical, horizontal, and diagonal arrows around the emission, before reaching the polaroid filters. The two polaroid filters either side of the flow cell are positioned in opposite orientations, such that one filter allows transmission of the vertical component of the fluorescence (**Parallel** orientation with respect to the plane polarised excitation light), whilst the other permits transmission of the horizontal component (**Perpendicular** orientation with respect to the plane of polarisation of the absorbed light). Fluorescence transmitted by the polaroid filter is detected by two PMTs (**PMT1** and **PMT2**).

**Figure 15** shows the restriction map of pGen001 a preferred recombinant vector according to the present invention.

**Figure 16** shows full sequence of pGen001 in FASTA format

**Figure 17** shows full sequence of pGen001 in GeneBank format

**Figure 18** represents data from Example 2 illustrating that Methyl methanesulfonate is strongly cytotoxic and genotoxic.

**Figure 19** represents data from Example 2 illustrating that Benzaldehyde is cytotoxic and genotoxic.

**Figure 20** provides Genotoxicity and cytotoxicity data from the Assay described in Example 2 including test concentration and limits of detection, with comparative data from other genotoxicity tests according to Example 2.

**Figure 21** provides extracted genotoxicity data comparing GreenScreen Assay and reported Ames Test results highlighting compounds requiring S9 metabolic activation for a positive Ames result according to Example 2.

**Figure 22** illustrates the Greenrack loading sequence according to Example 3.

**Figure 23** illustrates a microplate layout according to Example 3.

## **EXAMPLE 1**

The discovery of brighter cells carrying a mutant pWDH445 will be described. This brighter strain was compared with a typical pWDH445-bearing strain followed by phenotypic analysis of the new strain and molecular biological examination of the plasmid borne by these cells. Finally, the creation of brighter strains by basic genetic engineering is disclosed

## **1.2 MATERIALS & METHODS**

### **1.2.1 Strains and Plasmids**

#### **1.2.1.1 Strains**

*Saccharomyces cerevisiae* and *Escherichia coli* strains used in the Example are listed in Table 1, along with their respective genotypes.

**Table 1**

Strain	Organism	Genotype	Source
FF18984	<i>S. cerevisiae</i>	<i>MATa leu2-3, 112 lys2-1 his7-1 ura3-52</i>	F. Fabre
DH5 $\alpha$	<i>E. coli</i>	<i>SupE44 <math>\Delta</math>lacU169 (<math>\phi</math>80lacZ<math>\Delta</math>15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Laboratory collection

#### **1.2.1.2 Plasmids**

pWDH445 – is illustrated in **Figure 5** and corresponds to yEGFP-444 as disclosed in WO 97/44149 (e.g. see Figure 12)

### **1.2.2 Media**

#### **1.2.2.1 Sterilisation**

All media were prepared by dissolution of the components in distilled water before sterilisation by autoclaving for 20 minutes at 15 lbin<sup>-2</sup> unless otherwise stated. Alternatively, sterilisation was performed by filtration through 0.22  $\mu$ m diameter pore Millipore filters where autoclaving was inappropriate.

#### **1.2.2.2 Yeast media**

Bacto-agar was added to a final concentration (w/v) of 2% to each of the growth media (1.2.2.2.1 –1.2.2.2.4). Yeast extract, peptone, and agar were all obtained from Difco (Becton Dickinson, Sparks, MD 21152, USA).

#### 1.2.2.2.1 YPD (yeast extract, peptone, and dextrose)

Table 2

Component	% w/v	Final concentration gL <sup>-1</sup>
Bacto-yeast extract	1	10
Bacto-peptone	2	20
Dextrose (glucose)	2	20
*Bacto-agar	2	20)

\*Bacto-agar added in preparation of solid medium.

YPD was used as a rich growth medium to obtain good yeast cell yield, for unselective growth, and for selection of cells carrying antibiotic resistance by addition of antibiotic to the medium at the appropriate concentration. Glucose was diluted from a 40% stock (w/v) to give a final concentration of 2%, but was only added as required since it caramelises when autoclaved in YPD. Bacto-agar was added (2% w/v) in order to prepare solid medium.

#### 1.2.2.2.2 – YPG (synthetic dextrose)

Table 3 - YPG

Component	% w/v or v/v	Final concentration gL <sup>-1</sup> or mL <sup>-1</sup>
Bacto-yeast extract	1	10
Bacto-peptone	2	20
Glycerol	3 (v/v)	30 ml
*Bacto-agar	2	20

\*Bacto-agar added in preparation of solid medium.

YPG is a complex medium containing glycerol as a non-fermentable carbon source.  $\rho^-$  or “petite” mutants are respiratory deficient mutants that cannot use glycerol as a carbon source and thus cannot grow on YPG. Again the medium was autoclaved



before addition of the carbon source and Bacto-agar was added to 2% w/v for solid medium.

#### 1.2.2.2.3 - SD

**Table 4 -SD medium**

Component	% w/v	Final concentration gL <sup>-1</sup>
**Bacto-yeast nitrogen base	0.17	1.7
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.5	5
Dextrose (glucose)	2	20
*Bacto-agar	2	20

\*Bacto-agar is added in the preparation of solid medium.

\*\* Bacto-yeast nitrogen base was purchased without amino acids or ammonium sulphate. This allows control of the nitrogen source and facilitates the preparation of selective media. A 20x YNB stock comprising 34gL<sup>-1</sup> Bacto-yeast nitrogen base and 100 gL<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was stored in the dark at 4°C.

SD is a synthetic defined minimal medium used for selection of auxotrophic mutants by controlling the nutritional supplements provided (amino acids, nucleic acid bases etc.). Supplements primarily used in this work were adenine sulphate (Ade), L-histidine (His), L-leucine (Leu), L-lysine (Lys), L-tryptophan (Trp), and uracil (Ura). These were used as described (Sherman *et al.*, 1986) and as shown in **Table 2.2.5**. YNB was typically made as a 20x stock solution including ammonium sulphate, as above. In the preparation of SD, the YNB was diluted to its 1x working concentration. SD was supplemented with the appropriate amino acids and bases in the quantities shown below. YNB and all amino acids and bases were obtained from Sigma-Aldrich (Sigma Chemical Co, St. Louis, MO 63178, USA).

**Table 5 – SD Supplements**

Component	Working concentration mgL <sup>-1</sup>	Stock solution g/100 ml	Volume of stock mL <sup>-1</sup>
Adenine sulphate	20	0.2	10
Uracil	20	0.2	10
L-histidine	20	1.0	2
L-leucine	30	1.0	3
L-lysine	30	1.0	3
L-tryptophan	20	1.0	2

**1.2.2.2.4 – F1 medium (fermentation one)****Table 6 - F1 medium - salts stock (6.7x)**

Component	[x6.7] gL <sup>-1</sup>	Final conc. gL <sup>-1</sup>
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	20.88	3.13
KH <sub>2</sub> PO <sub>4</sub>	13.33	2.00
MgSO <sub>4</sub> . 7H <sub>2</sub> O	3.66	0.55
CaCl <sub>2</sub> . 2H <sub>2</sub> O	0.60	0.09
NaCl	0.67	0.10

**Table 7 - F1 medium - trace elements stock (10000x)**

Component	[x10000] gL <sup>-1</sup>	Final conc. mgL <sup>-1</sup>
ZnSO <sub>4</sub> . 7H <sub>2</sub> O	0.7	0.07
FeCl <sub>3</sub> . 6H <sub>2</sub> O	0.5	0.05
CuSO <sub>4</sub> . 5H <sub>2</sub> O	0.1	0.01
H <sub>3</sub> BO <sub>3</sub>	0.1	0.01
KI	0.1	0.01

**Table 8 - F1 medium – vitamins stock (600x)**

Component	[x600] gL <sup>-1</sup>	Final conc. mgL <sup>-1</sup>
Inositol	37.2	62
Thiamine/HCl	8.4	14
Pyridoxine	2.4	4
Ca-pantothenate	2.4	4
Biotin	0.18	0.3

F1 medium is a defined minimal medium used in fermentation [Brown *et al*, 1981, adapted by Walmsley *et al.*, 1983] that has a particularly low background autofluorescence. Concentrated stocks of salts, trace elements and iron (III) chloride were autoclaved, stored separately, and diluted into sterile water upon requirement. A concentrated vitamin stock was prepared in sterile water, filter sterilised by syringe and sterile 0.20 µm pore filter (Sartorius, Gottingen, Germany), and stored in aliquots at -20°C. Vitamins were then added subsequent to autoclaving of F1, to avoid their denaturation. All F1 was stored at 4°C in the absence of glucose.

A subsequent variant form of F1 was employed with 50% less inositol than quoted in the vitamins stock **Table 8** in order to lessen the effects of flocculation in troublesome yeast strains. This reduced inositol F1 was further modified by producing it in phosphate buffer at pH6 (as described in Varley, fourth edition, 1967) instead of in water. The buffered medium was created by mixing 0.067 M solutions of KH<sub>2</sub>PO<sub>4</sub> and Na<sub>2</sub>HPO<sub>4</sub>, or concentrated stocks thereof (see **Table 9**), before addition of salts, trace elements, iron chloride, and nutritional supplements. The buffer mix was autoclaved and allowed to cool down to room temperature before addition of vitamins.

**Table 9 - Phosphate buffer for F1 medium**

Component	Volume of 1/15 M PO <sub>4</sub> <sup>2-</sup> /100 ml ml
Na <sub>2</sub> HPO <sub>4</sub>	12.2
KH <sub>2</sub> PO <sub>4</sub>	87.8

### 1.2.2.3 Bacterial media

The only bacterium used was *E. coli* which was grown on 3 different media; LB, SOB, and SOC.

#### 1.2.2.3.1 – LB medium

**Table 10 - LB Medium**

Component	Concentration gL <sup>-1</sup>
Bacto-tryptone	10
Bacto-yeast extract	5
NaCl	10

Luria-Bertani medium (LB) for broth cultures was prepared as described [Sambrook *et al.*, second edition, 1989] and LB broth plus 2% Bacto-agar was used for solid cultures.

#### 1.2.2.3.2 - SOB

**Table 11 - SOB**

Component	Final concentration gL <sup>-1</sup>
Bacto-tryptone	20
Bacto-yeast extract	5
NaCl	0.5

SOB was prepared as described in Sambrook *et al.* (second edition, 1989). After autoclaving the above components in 950 ml of water, 10 ml of 250 mM KCl were added before shifting the pH to 7 with 5 N NaOH. Immediately prior to use, 5 ml of 2 M MgCl<sub>2</sub> were added.

#### 1.2.2.3.3 - SOC

SOC is identical to SOB except for the inclusion of 20 mM glucose after autoclaving.

### **1.2.3 Antibiotics**

#### **1.2.3.1 - Ampicillin**

Ampicillin was prepared in aqueous solution at a stock concentration of  $10 \text{ mgml}^{-1}$  and used at working concentrations of up to  $100 \text{ } \mu\text{gml}^{-1}$ . Ampicillin stock solutions were stored in aliquots of 1 ml at  $-20^{\circ}\text{C}$ .

#### **1.2.3.2 - Geneticin (G418)**

G418 disulphate salt was prepared in aqueous solution as a stock solution at the concentration of  $20 \text{ mgml}^{-1}$  and used at a working concentration of  $200 \text{ } \mu\text{g/ml}$ . The G418 stock solution was stored at  $4^{\circ}\text{C}$ .

#### **1.2.3.3 - Kanamycin**

Kanamycin was also prepared in aqueous solution, at a stock concentration of  $50 \text{ mgml}^{-1}$  and used at a working concentration of  $50 \text{ } \mu\text{gml}^{-1}$ . Kanamycin stock solutions were stored at  $-20^{\circ}\text{C}$ .

### **1.2.4 Standard solutions & Buffers**

These are listed in alphabetical order for ease of navigation and those chemicals deemed to be carcinogenic or potentially carcinogenic are marked with an asterisk (\*).

**“Crushing” buffer:** 20 mM Tris-HCl pH 7.5, 0.1 M NaCl, 1 mM EDTA

**EDTA:** 0.5 M EDTA (ethylenediaminetetra-acetic acid) pH 8.0 (pH adjusted to 8.0 by the addition of sodium hydroxide pellets)

**Ethidium bromide\*:** A stock solution at a concentration of  $10 \text{ mgml}^{-1}$  was prepared in sterile distilled water and stored in the dark at  $4^{\circ}\text{C}$ . It was diluted to a working concentration of  $0.5 \text{ } \mu\text{gml}^{-1}$  as required.

**“Extraction” buffer:** 20 mM Tris-HCl pH 7.0; 0.1M NaCl; 1 mM EDTA

**Lithium acetate:** 1 M lithium acetate (This was a 10x concentrated stock solution for lithium acetate transformation).

**10x Loading dye:** 25% w/v Ficoll (type 400, Pharmacia); 0.25% w/v bromophenol blue; 0.25% w/v xylene cyanol FF

**Lysing solution:** 0.25 M Tris-HCl pH 7.5; 25 mM EDTA; 2.5% SDS

**Methylmethane sulphonate (MMS, methanesulphonic acid methyl ester)\*:** Liquid stock solution diluted as required. Fresh diluted stock solutions were prepared for each experiment; 5  $\mu$ l 100% MMS and 995  $\mu$ l sterile water for a 0.5% (v/v) stock solution; **NB** All MMS concentrations quoted throughout this thesis are v/v percentages.

**Polyethylene glycol:** 50% w/v PEG 4000

**Phenylmethanesulphonyl fluoride (PMSF):** 0.1 M PMSF; Stored in isopropanol at 4°C.

**Potassium acetate:** 5 M potassium acetate

**Restriction endonuclease buffers:** The buffers recommended by the manufacturers were used unless otherwise stated.

**RNAase:** 10 mgml<sup>-1</sup> RNAase A; Prepared in a similar way to the protocol described in Sambrook et al. (second edition, 1989). 10 mgml<sup>-1</sup> RNase A was dissolved in 0.01 M potassium acetate (pH 5.2) and incubated at 100°C for 15 minutes. It was allowed to cool slowly to room temperature before the pH was adjusted by the addition of 0.1 volumes of 1M Tris-HCl (pH 7.5).

**S-buffer:** 10 mM NaHPO<sub>4</sub> pH7.2; 10 mM EDTA; 50 mM 2-mercaptoethanol; 10 mgml<sup>-1</sup> Novozyme

**Sodium azide:** 2% w/v sodium azide (NaN<sub>3</sub>) stock solution

**Sodium chloride:** 1 M NaCl

**Sodium dodecyl sulphate (SDS):** 10% w/v SDS; The stock solution did not require sterilisation.

**Sodium hydroxide:** 10 N stock solution; Diluted as required and did not require sterilisation.

**Solution I:** 50 mM glucose; 25 mM Tris-HCl (pH 7.5); 10 mM EDTA (pH 8.0);

**Solution II:** 0.2 N NaOH; 1% SDS; Freshly prepared from concentrated stock solutions as required.

**Solution III:** 60 ml 5 M potassium acetate; 11.5 ml glacial acetic acid; 28.5 ml distilled water

**TB:** 1.62 g PIPES; 12.5 ml 1 M KCl; 7.5 ml 1 M CaCl<sub>2</sub>; 450 ml water; The pH was adjusted to 6.7 with KOH, made up to 495 ml with water, then 5.45 g Mn(II)-tetrahydrate added before filter sterilising through 0.45  $\mu$ m pore filter unit.

**5x TBE (Tris-borate, EDTA):** 54g Tris base; 27.3g boric acid; 20 ml 0.5 M EDTA; Distilled water to make up to 1 L.

**TE (Tris-EDTA):** 100 mM Tris; 10 mM EDTA; 10x stock diluted to working concentration.

**Tris-HCl pH 7.5:** 1 M Tris; pH adjusted to 7.5 by drop-wise addition of HCl.

### **1.2.5 Experimental Procedures**

#### **1.2.5.1 - Growth Conditions**

##### **1.2.5.1.1 - *S. cerevisiae***

All yeast cultures grown on solid medium were statically incubated at 30°C in an Heraeus Electronic B5050 E temperature controlled incubator (Heraeus Instruments GmbH, Hanau, Germany). Liquid phase cultures were grown either in 250 ml conical flasks or 15 ml plastic centrifuge tubes. The temperatures used were 25°C or 30°C, and broth cultures were always shaken vigorously and continuously. Flask cultures were shaken in a Model G25 New Brunswick Scientific orbital incubator shaker (New Brunswick Scientific Co. Inc., Edison, New Jersey, USA). Centrifuge tubes were incubated either in a test tube rack fastened into the New Brunswick orbital shaker or in specially adapted Model G76 Gyrotory® Water Bath Shakers (New Brunswick Scientific Co. Inc.).

Solid phase cultures were maintained at 4°C as stock cultures for up to 6 months. These were used as sources of inocula for other agar plate cultures and liquid phase starter cultures. Stock cultures were also maintained at -80°C after growing 1.5 ml liquid cultures overnight and adding 1.5 ml of 30% glycerol (final 15% glycerol). The resultant 3 ml culture was split into 2 aliquots of 1.5 ml in sterile 2 ml screw-capped cryogenic vials (Nalgene, Nalge Co, Rochester, NY, USA).

##### **1.2.5.1.2 - *E. coli***

Solid phase *E. coli* cultures were grown under temperature controlled conditions at 37°C in a temperature controlled incubator. Liquid cultures were grown with vigorous shaking at 37°C in a Model G25 New Brunswick Scientific orbital incubator shaker. Solid phase cultures were maintained at 4°C but were generally not re-used.

### **1.2.5.2 – Transformation protocols**

#### **1.2.5.2.1 – Lithium acetate transformation of yeast with plasmid DNA**

Transformation of *S. cerevisiae* with plasmid DNA was performed by a rapid lithium acetate/PEG/single-stranded DNA method, adapted from that described by Gietz *et al.*, 1995. An initial “starter” culture of the appropriate yeast strain was prepared by scraping a loopful of frozen stock culture or by picking a portion of a colony with an inoculating loop from an agar plate stock culture, and inoculating 50 ml of YPD in a 250 ml conical flask. This starter culture was incubated overnight (~16 hours) at 30°C with shaking. A 10 µl aliquot was removed and diluted to 1 ml in a 1 ml plastic cuvette. A second cuvette was filled with 10 µl of fresh YPD medium diluted to 1 ml, to act as a blank for optical density (OD) measurement. Using a Cecil Instruments CE505 Double Beam Ultraviolet Spectrophotometer with the wavelength set to 600 nm, the absorbance of the blank was set to zero before reading the OD of the diluted culture. Using the known OD of the starter culture, the culture was then sub-cultured to the required starting OD of approximately 0.2 in 50 ml of fresh YPD in a 250 ml conical flask. The sub-culture was incubated for 3 to 4 hours to allow approximately 2 complete generation times, to ensure that a large proportion of the culture is in the early to mid- exponential phase of growth required for transformation.

30 ml of the sub-culture were transferred to 50 ml Falcon tubes, and the cells harvested by centrifugation in an Eppendorf Centrifuge 5403 (Eppendorf, Hamburg, Germany) at 4000 rpm for 4 minutes. The supernatant was discarded and the cell pellet was washed (resuspended and then harvested again, followed by removal of the supernatant) twice in 10 ml of sterile water, harvesting by centrifugation as before. The resulting cell pellet was this time resuspended in 1 ml of sterile water and transferred to a sterile 1.5 ml microfuge tube. The cells were harvested in the microfuge tube by centrifugation in an Eppendorf Centrifuge 5414 (microfuge) for 30 seconds. The supernatant was discarded and the pellet washed in 1 ml of 0.1 M lithium acetate/1x TE (1x TE is 10 mM Tris-Cl pH7.5, 1 mM EDTA pH8.0), the supernatant removed and the pellet resuspended in 0.5 ml of 0.1 M lithium acetate/1x TE.



Sheared salmon sperm DNA, prepared by sonication, was boiled for 10 minutes in a boiling water bath to melt the double-stranded DNA into single-stranded "carrier" DNA. After boiling, the carrier DNA was immediately transferred to ice for 2 minutes. As long as single-stranded carrier DNA was immediately moved onto ice incubation and was re-frozen shortly after boiling, the salmon sperm DNA was only boiled every 4 or 5 times it was defrosted.

50  $\mu$ l aliquots of cells in 0.1 M lithium acetate/1x TE were transferred to sterile microfuge tubes. To each aliquot were added  $\sim$ 1  $\mu$ g of plasmid DNA,  $\sim$ 20  $\mu$ g of single-stranded carrier DNA, and 300  $\mu$ l of 40% PEG/1x TE/0.1 M lithium acetate. The contents of each microfuge tube were mixed carefully and thoroughly by pipette, before transferral of the tubes to a shaking incubator for 30 minutes incubation at 30°C with shaking. After 30 minutes at 30°C, the tubes were transferred to a water bath with the temperature set to 42°C. This heat shock incubation lasted for 15 minutes, although this could be varied depending on strain tolerance of such stress.

After the heat shock treatment, each transformation mix was further diluted with 0.5 ml of sterile water before harvesting the cells and removal of all traces of the PEG containing supernatant. To ensure that all PEG was removed, the cells were further washed carefully with 1x TE before eventually resuspending the cell pellets in 1 ml of fresh YPD by pipette. Cells were given 2 hours at 30°C with shaking, or  $\sim$ 16 hours overnight at 4°C to recover in YPD.

For selection of antibiotic resistant transformants, 150  $\mu$ l of the 1 ml YPD culture of transformed cells were pipetted onto YPD-agar incorporating the selection antibiotic. Cells were spread onto the agar using an ethanol-flamed glass spreader under aseptic conditions and the agar plates allowed to dry in a laminar flow cabinet, before incubating at 30°C for 3 to 5 days.

If the method of transformant selection was suppression of auxotrophy, then the cells from the 1 ml YPD culture were harvested and the YPD supernatant removed. All traces of YPD had to be removed since even small amounts can permit background growth of non-transformants. Hence, after removal of the YPD supernatant, the cell

pellets were washed with 1x TE before finally resuspending in 1 ml 1x TE. 150 µl of each 1x TE suspension were transferred to SD-agar plates incorporating the required nutritional supplements except one for which the untransformed cells were auxotrophic, to allow for selection of cells that have become prototrophic for the missing nutrient through transformation. Again the cell suspensions were spread using a glass spreader with aseptic technique and allowed to dry in a laminar flow cabinet before incubation at 30°C for 3 to 5 days.

All transformants (auxotrophic or antibiotic selected) were replica plated onto sets of SD-agar plates, each with one required nutrient missing. This was performed in order to check that transformants had the correct marker gene phenotype. True transformants should grow on plates containing all the nutrients required by untransformed cells, or on plates lacking only the nutrient for which transformants acquire prototrophy (due to transformation).

#### **1.2.5.2.2 - Transformation of *E. coli* with plasmid DNA**

##### **1.2.5.2.2.1 - Preparation of competent *E. coli* cells**

Competent *E. coli* cells were prepared by the following protocol (J. Schmuckli-Maurer, Institute of Cell Biology, University of Bern, personal communication). A starter culture of *E. coli* cells (DH5α strain) was prepared in a volume of typically ~5 ml of LB broth in a universal tube. This culture was incubated overnight at 37°C with shaking and then sub-cultured into a 2 L flask containing 250 ml of SOB medium, to give an initial OD<sub>600nm</sub> of approximately 0.1. The sub-culture was incubated with shaking at room temperature for 18 to 24 hours before being incubated on ice for 10 minutes. After ice incubation the cells were harvested by centrifugation at 8000 rpm in an Eppendorf Centrifuge 5403. The supernatant was discarded and the pellet resuspended in 80 ml of TB buffer and the tubes incubated on ice for another 10 minutes. Again the cells were harvested and the TB supernatant discarded and this time the pellets resuspended in 20 ml of TB buffer. To the 20 ml TB buffer cell suspension, 1.4 ml of DMSO were added and mixed gently before another 10 minutes ice incubation. 1 ml aliquots of the competent cells in 1.5 ml microfuge tubes were snap-frozen in liquid nitrogen and stored in a -80°C freezer.

#### **1.2.5.2.2.2 - Transformation of competent *E. coli* cells with plasmid DNA**

The following *E. coli* transformation protocol is taken from Inoue and co-workers (1990). Aliquots of competent cells stored at -80°C were allowed to defrost at room temperature. 1.5 ml microfuge tubes were pre-cooled before the addition of ~100 ng of plasmid DNA. 200 µl of defrosted competent cells were added to the DNA and incubated on ice for 30 minutes. After 30 minutes, the *E. coli*/DNA mixtures were transferred to a 42°C water bath for 30 seconds for heat shock treatment. Immediately after the heat shock, the tubes were cooled by ice incubation. To each transformation tube, 0.8 ml of SOC medium (though LB with 2% glucose added suffices) were added and these suspensions were shaken for 1 hour at 37°C.

Transformed *E. coli* were antibiotic resistant, so the SOC or LB used for recovery of the cells at 37°C did not have to be removed to limit background growth. 100 to 150 µl of transformed cells were spread onto LB-agar incorporating the antibiotic required for selection, using aseptic technique. The agar plates were allowed to dry in a laminar flow cabinet and were then incubated at 37°C overnight.

#### **1.2.5.3 – Isolation of plasmid DNA**

##### **1.2.5.3.1 - Preparation of plasmid DNA from yeast cells (yeast “mini-prep”)**

The protocol described here for the preparation of plasmid DNA from yeast cells was provided by J. Schmuckli-Maurer (University of Bern) in a personal communication. Yeast scraped from a plate was resuspended in 1 ml of sterile water in a microfuge tube. The OD<sub>600nm</sub> of this suspension was adjusted to between 2 and 5 units. Alternatively, cells were taken from a small (1-5 ml) overnight culture. Cells were harvested by centrifugation and the supernatant discarded. Cell pellets were resuspended in 0.5 ml of S buffer and incubated at 37°C for 30 minutes. 100 µl of lysing solution were added to each cell suspension and mixed by vortexing and then incubated at 65°C for 30 minutes. 96 µl of 5 M potassium acetate (pH 5.2) were added to each tube before incubating on ice for 10 minutes. Cell debris was precipitated by centrifugation in a microfuge for 10 minutes and the resulting supernatants were removed to fresh sterile microfuge tubes. DNA was precipitated from the supernatants by the addition of 800 µl of cold ethanol, 10 minutes ice incubation and then centrifugation in a microfuge for 10 minutes. The ethanolic

supernatants were discarded and traces of ethanol removed by aspiration. Pellets were washed with 500  $\mu$ l of 70% ethanol and then allowed to air-dry. Dry pellets were resuspended in 40  $\mu$ l of sterile water and 5  $\mu$ l (~100 ng) of this preparation were used to transform competent *E. coli* cells. Remaining DNA was stored at -20°C.

#### **1.2.5.3.2 - Preparation of plasmid DNA from *E. coli* cells (bacterial "mini-preps")**

Small-scale preparation of plasmid DNA from *E. coli* was performed by alkaline lysis as described in Sambrook *et al.* (second edition, 1989). Single bacterial colonies were transferred to individual sterile 15 ml test tubes containing 2 ml of LB broth medium plus the appropriate antibiotic for the maintenance of plasmid selection. The cultures were incubated overnight at 37°C with vigorous shaking. 1.5 ml aliquots were taken from each culture and transferred to 1.5 ml microfuge tubes. Cells were harvested by centrifugation in a microfuge for 30 seconds and then the supernatant removed by aspiration (using a disposable pipette tip attached to a vacuum line) to leave the pellet as dry as possible.

The bacterial pellets were resuspended in 100  $\mu$ l of ice-cold solution I by vigorous mixing. 200  $\mu$ l of freshly prepared solution II were added to each suspension and mixed together by rapid inversion of the tubes. The tubes were chilled on ice whilst 150  $\mu$ l of solution III were added to each mixture. The contents of the tubes were mixed whilst inverted to ensure appropriate mixing before incubating on ice for 5 minutes. DNA was precipitated by addition of 2 volumes of ethanol at room temperature and vortexed to mix. This was allowed to stand for 2 minutes before harvesting the DNA by centrifugation in a microfuge for 5 minutes. The supernatants were removed by aspiration (tip and vacuum line, as before) and the pellets were allowed to air-dry, prior to rinsing the DNA pellets with ice-cold 70% ethanol. The supernatants were again aspirated off, taking care not to dislodge the pellets. Pellets were allowed to air-dry again before being resuspended in 50  $\mu$ l of TE containing 20  $\mu$ gml<sup>-1</sup> DNase-free RNAase. DNA preparations were stored at -20°C.

#### **1.2.5.4 – Manipulation of DNA**

##### **1.2.5.4.1 - Restriction enzyme cleavage of plasmid DNA**

Enzymes and buffers were obtained from Boehringer Mannheim (now Roche Diagnostics Ltd., Lewes, UK) and digests were carried out according to the manufacturer's instructions. A typical restriction enzyme digest was carried out as follows for a 10  $\mu$ l reaction: 1  $\mu$ l sample DNA (approximately 25 to 75 ng) isolated from *E. coli* by method 1.2.5.3.2, 1  $\mu$ l of the appropriate enzyme buffer, 1  $\mu$ l enzyme (~10 units), and sterile water to make the volume up to 10  $\mu$ l. The reaction was incubated at 37°C for between 1 and 2 hours or until the DNA was digested.

##### **1.2.5.4.2 - Ligation of "sticky-ended" DNA termini**

Plasmid DNA was cut by digestion with restriction endonuclease, as described above, to leave single-stranded overhangs or "sticky ends". The success of the digestion can be checked by agarose gel electrophoresis of a small sample of restricted DNA. Digested DNA was incubated at 65°C for 20 minutes in order to inactivate most restriction enzymes. This DNA was then diluted to the appropriate concentration (~100  $\mu$ gml<sup>-1</sup>) and chilled to 0°C. In order to re-join the ends of the plasmid without insertion of foreign DNA, the ligation reaction was set up as follows (as described in Sambrook *et al.*, 1989). Approximately 0.1  $\mu$ g of DNA was transferred to a fresh sterile microfuge tube. To this were added 2  $\mu$ l of 10x bacteriophage T4 DNA ligase buffer and 0.1 units of bacteriophage T4 DNA ligase. The reaction was made up to a final volume of 20  $\mu$ l with the addition of sterile water. Reactions were typically incubated at 15 or 16°C (controlled temperature water bath in 4°C cold room) overnight. Bacteriophage T4 ligase and its respective buffer were from Boehringer Mannheim.

##### **1.2.5.4.3 - Agarose gel electrophoresis**

Agarose gel electrophoresis was carried out using the standard procedure [Sambrook *et al.*, 1989]. 1% agarose gels were prepared with running buffer 0.5x TBE with 0.5  $\mu$ gml<sup>-1</sup> ethidium bromide. Lambda DNA digested with *Hind* III and *Eco* RI, and a 500 base pair (bp) ladder (500-5000 bp) were used as molecular weight markers. Both types of marker DNA, along with the restriction enzymes and buffers, were from Boehringer Mannheim.

### **1.2.5.5 – Fluorescence detection and quantitation**

#### **1.2.5.5.1 - Fluorescence assays with cell extracts**

The assessment of GFP fluorescence in cell extracts was performed essentially as described in Walmsley *et al.*, 1997 (Yeast 13 p1535-1545). A stationary phase culture of cells grown in SD medium was used as the inoculum source. 15 µl aliquots of cells were inoculated into 1.5 ml of SD in 15 ml test tubes. Half of the tubes were then supplemented with 0.01% MMS (the remaining tubes left as controls). The tubes were incubated at 25°C (accurate folding of the S65T derivative of GFP is temperature sensitive) for 16 hours in an orbital shaker incubator at 120 rpm, or in a water bath shaker. After 16 hours the cultures were adjusted to 0.02% NaN<sub>3</sub> to inhibit respiration and incubated on ice for 90 minutes, maintaining agitation. The cells were then transferred to 1.5 ml microfuge tubes, harvested by centrifugation (10s), washed twice in sterile distilled water, then washed in 1 ml of “extraction” buffer (20 mM Tris-Cl pH 7.5, 0.1 M NaCl, and 1 mM EDTA). After harvesting the cells by centrifugation, the supernatant was aspirated off and the cell pellet resuspended in 250 µl “crushing” buffer (20 mM Tris-HCL, pH 7.5, 0.1 M NaCl, and 1 mM PMSF). 250 µl of 400-600 nm diameter glass beads were added to the tubes of cells that were then placed in a BIO101 Fastprep FP120 (Savant, purchased from Anachem Ltd, Luton, UK) on speed 4 for 30 seconds to mechanically disrupt the cells. The tubes were incubated on ice for 1 minute and then returned to the Fastprep for another 30 seconds. This process was repeated such that each tube had undergone 3 periods in the Fastprep separated by 1 minute ice incubations. Following centrifugation for 30s, the supernatant was transferred to a clean tube. The pellet of beads was washed in a further 250 µl of crushing buffer and this was added to the supernatant from the previous extraction. The extract was adjusted to 0.1 M Tris, pH 11 by the addition of 1 M Tris base, 200 µl of the pooled extract plus 2.8 ml of water was then transferred to an acrylic cuvette (Sarstedt Ltd, Numbrecht, Germany).

#### **1.2.5.5.2 - Fluorescence assays with intact yeast cells**

Starter cultures were initiated by inoculating 1.5 ml of F1 medium (plus appropriate nutritional supplements and 2% glucose) in sterile 15 ml polythene centrifuge tubes with small portions of colonies, picked by inoculating loop from stock plates. These

starter cultures were grown for up to 24 hours at 30°C with shaking (at 120 rpm) and were used as the source of inoculum for the assay cultures. Cells were inoculated into 1.5 ml F1 cultures in 15 ml polythene centrifuge tubes to give an initial OD<sub>600nm</sub> of ~0.1 (typically a 10-15 µl inoculum per 1.5 ml culture). The lids of the tubes were left one-quarter unscrewed, but held in place by masking tape, to ensure maximum oxygenation for GFP maturation. Half of the tubes were treated with 0.005% methyl methanesulphonate (MMS, methanesulphonic acid methyl ester) and half left as unchallenged controls. MMS was purchased as a liquid and this stock solution was taken to be 100%. This was diluted to a 0.5% stock in small aliquots as required and then diluted 1:100 for the assay cultures (15 µl of 0.5% stock per 1.5 ml culture). Assay cultures were incubated at 25°C (30°C when the S65T-GFP was replaced by yEGFP, which is more heat stable) in shaking water bath incubators for 14-16 hours. Each of the 1.5 ml cultures was transferred to a 4-window acrylic cuvette (Sarstedt Ltd, Numbrecht, Germany) and diluted with 1 ml of sterile water before measurement. For the measurement of intact yeast cell fluorescence of cells grown in YPD or SD, the cells were washed twice in sterile water to remove traces of autofluorescent medium and resuspended in 1.5 ml of sterile water. Washed cells were then transferred directly to 4-window acrylic cuvettes containing 1 ml of sterile distilled water. 4-window cuvettes were necessary since measurement of fluorescence emission is performed perpendicular to the path of excitation light.

#### **1.2.5.5.3 - Fluorescence measurements and “Brightness” calculation**

Fluorescence measurements were performed with a Perkin-Elmer LS50B Fluorescence Spectrometer (Perkin-Elmer Ltd., Beaconsfield, UK). The excitation and emission wavelengths were set to 488 nm and 511 nm respectively, with a slit width of 10 nm, for the S65T GFP derivative- expressing reporter. For the yEGFP expressing reporter, the excitation and emission wavelengths were set to 490 nm and 518 nm respectively (due to the altered fluorescence characteristics of yEGFP), with 5 nm slit widths. To account for differences in cell numbers between YPD or SD assay cultures, the OD<sub>600nm</sub> was recorded for each cuvette. Similarly, in order to measure protein extraction efficiency, light absorption at 280 nm (OD<sub>280nm</sub>) was measured for crude cell extracts. Both of these measurements used a Cecil Instruments CE505 double beam ultraviolet spectrophotometer. Acrylic cuvettes were essential for the

latter measurements since they permit greater transmission of ultraviolet (UV) light. Cell density estimations for F1 medium cultures were made by measuring light scattering (nephelometry) using the LS50B luminescence spectrometer, with both excitation and emission wavelengths at 600 nm and slit widths of 2.5 nm (Neph600). The fluorescence values obtained from the fluorometer were then divided by the absorption/scatter readings to give the "brightness value", an arbitrary unit which is independent of sample concentration, though varies with different fluorometers. The y-axis on uncorrected fluorescence scans gives raw data in the form of machine defined fluorescence intensity ("INT") units.

#### **1.2.5.5.4 – Induction ratio**

The induction ratio is used to calculate a "signal-to-noise ratio" with respect to the GFP signal. All detected signals are not pure GFP signals, but incorporate contaminating background fluorescence signals (autofluorescence). Autofluorescence significantly varies with the changing growth phase of a culture (see Chapter 5) and is dependent on the strain background. Hence, in order to be able to compare the signal-to-noise ratios from cultures of the same strain in different growth phases or from cultures of different strains, it is necessary to remove the variable autofluorescence component from the brightness values.

Firstly, several terms (used throughout this thesis) must be defined:

"U" is the brightness value from untransformed cells *i.e.* cells not bearing a reporter plasmid (or other plasmid where specified), that have not been exposed to a genotoxin such as MMS, and as such this value represents the uninduced autofluorescence.

"U MMS" is the brightness value from untransformed cells that have been exposed to a genotoxin (in this case, MMS). This value represents the genotoxin-induced level of autofluorescence, often larger than that of uninduced autofluorescence.

"T" is the brightness value from cells transformed with one of the *RAD54*-GFP reporter plasmids (or other plasmid where specified) that have not been exposed to a genotoxin. This value represents the constitutive level of GFP fluorescence due to



continual low-level expression from the *RAD54* promoter, and also incorporates the uninduced autofluorescence.

“T MMS” is the brightness value obtained from cells transformed with a plasmid that have been exposed to a genotoxin (again, MMS in this case). T MMS represents a combination of the GFP signal due to damage-induced up-regulation of expression from the *RAD54* promoter, the constitutive GFP signal, and the damage-induced autofluorescence signal.

For a true signal-to-noise ratio based solely on the signal from GFP, the two parameters of interest are the GFP signal due to the damage-induced response of *RAD54* and the constitutive GFP response in the absence of damage. The constitutive signal is obtained by subtracting the uninduced autofluorescence from T (*i.e.* T – U) and the induced GFP signal is calculated by subtracting the induced autofluorescence from T MMS (*i.e.* T MMS – U MMS). (T – U) gives the constitutive signal or C value, whereas (T MMS – U MMS) provides the induced value or I value. The induction ratio is a simple ratio of I to C and can be expressed thus:

$$\text{Induction Ratio} = \frac{(\text{T MMS} - \text{U MMS})}{(\text{T} - \text{U})} \quad \frac{(\text{GFP signal})}{(\text{GFP noise})}$$

#### 1.2.5.5.5 - Flow-through fluorescence detector

The development of a continuous-flow detector for the measurement of GFP signal from a yeast culture has been described previously [Knight *et al.*, 1999, Measurement Science and Technology, 10: 211, 217] and adapted for the fluorescence polarisation technique [Knight *et al.*, 2000a, The Analyst, 125: 499-506]. **Figure 13** shows a schematic diagram of the basic layout of the instrumentation developed with a fluorescence flow cell, through which the yeast culture or GFP extract is circulated by use of a Gilson Minipuls 3 peristaltic pump (purchased from Anachem Ltd, Luton, UK). The 488 nm excitation was provided by an air-cooled 40 mW argon ion laser (LG Laser Graphics GmbH, Dieberg, Germany), reduced to 5 mW by filtering. A photomultiplier tube (PMT) was used as the fluorescence detector and a silicon photodiode (SPD) as the scattered light detector for nephelometric measurement of

the cell density. The flow cell, PMT, SPD, and associated electronics were housed in a light-tight box (in duplicate in **Figure 13**). The PMT is positioned to one side of the flow cell such that fluorescence is detected at 90° to the path of the excitation light. The SPD is situated at the other side of the flow cell from the light source in the path of the excitation light, or to one side of the flow cell in the same way as the PMT, since scattered light can be detected in either orientation. Data acquisition and manipulation was performed in real time on a personal computer *via* a 12-bit analogue to digital converter (ADC) and associated software (Pico Technology Ltd., Cambridge, UK).

#### **2.5.5.6 - Fluorescence polarisation**

**Figure 14** shows the detection of fluorescence polarisation using the flow-through fluorescence detector. Polarised laser light (polarisation represented by vertical arrows) is used to excite the sample circulating through the flow cell. In this cartoon, fluorescence is detected at 90° on either side of the flow cell with a single polaroid filter between the flow cell and each PMT. Fluorescence from the sample in the flow cell is less polarised than the excitation light (represented by vertical, horizontal, and diagonal arrows). The polaroid filters to either side of the flow cell are arranged in opposite orientations, such that one filter allows the vertical component of the fluorescence to pass through (parallel orientation, with respect to the plane of polarisation of the incident light), while the other permits transmission of only the horizontal component of the fluorescence (perpendicular orientation, with respect to the plane of polarisation of the incident light).

The degree of fluorescence polarisation ( $P$ ) measured in this work was defined as:

$$P = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$$

where  $I_{\parallel}$  is the fluorescence intensity measured polarised parallel to the absorbed plane-polarised radiation, and  $I_{\perp}$  is that perpendicular to the absorbed radiation.  $P$  is a dimensionless parameter and is not dependent on the intensity of the emitted light or on the concentration of the fluorophore. For the discrimination of GFP signal from

that of autofluorescence, calculation of the  $(I_{\parallel} - I_{\perp})$  term is sufficient, since this term tends to zero for molecules emitting relatively unpolarised fluorescence (for example, small molecules like fluorescein), whilst being relatively large for molecules emitting fluorescence that exhibits significant polarisation.

### **1.3 RESULTS**

#### **1.3.1 Brighter FF18984 transformants bearing pWDH445**

##### **1.3.1.1 - Spontaneous generation of bright pWDH445-bearing FF18984 cells**

After the production of new pWDH445-containing strains, transformants screened by marker selection were assayed for a change in brightness in response to MMS, to ensure the presence of functional pWDH445.

Sporadically, pWDH445 transformants (transformed using experimental procedure 1.2.5.2) were identified which were significantly brighter in response to MMS than typical transformants. **Figure 1** shows the average normal brightness for 3 replicates of FF18984 cells carrying pWDH445 (darker bars) and the average brightness for 3 replicates of brighter FF18984 cells carrying pWDH445 (lighter bars). In this example, 'T' (transformed cells with no MMS) was 12 fold brighter in the new variant FF18984 cells than the typical signal level, and 'T MMS' was 9 fold brighter. The enhanced brightness in the variant cells actually led to a decrease in the induction ratio from 7.72 to 3.27. However, the ease of detection of the variant cells with enhanced signal output made them attractive for the development of ever-brighter reporters.

##### **1.3.1.2 - There is a link between enhanced signal output and geneticin resistance**

The bright variants discussed above were first subjected to phenotypic analysis to confirm that they were indeed FF18984 transformants. Strains were re-plated onto media excluding uracil to check for the presence of the pWDH445. This was the favoured method of transformant selection to minimise the use of antibiotics (in this case G418). Both normal and variant transformed cells grew on SD medium lacking uracil. Cells were replica-plated onto SD medium lacking one of leucine, lysine, or histidine, and once more both normal and variant strains responded accordingly: *i.e.* they were unable to grow. This suggested that both normal and variant cells had the correct genetic background for FF18984.

The cells bearing pWDH445 were then tested for resistance to G418, which is conferred by the *kanMX* module carried on pWDH445. Cells of typical brightness were G418 resistant and hence able to grow on G418 (200  $\mu\text{gml}^{-1}$ ) containing YPD

plates. However, bright variant cells were unable to grow on such medium, despite the previous confirmation of the presence of pWDH445 by growth on uracil-deficient medium. This suggested that mutation or rearrangement of the reporter plasmid had occurred in the variant cells.

#### **1.3.1.3 - The *RAD54*-GFP cassette has no gross rearrangements**

In order to test the hypothesis that the plasmid had undergone a rearrangement or mutation, it was necessary to isolate pWDH445 from both normal cells and variant cells. Plasmid was prepared from yeast as described in experimental procedure 1.2.5.2.2 and the DNA pellet was dissolved in 40  $\mu$ l of sterile water. 5  $\mu$ l of each plasmid preparation was used to transform DH5 $\alpha$  *E. coli* cells by the protocol described in method 1.2.5.2.2. *E. coli* transformed with the plasmids prepared from yeast were selected by growth on LB agar containing 80  $\mu$ gml<sup>-1</sup> ampicillin. Transformant cultures and plasmid preparations were made as described in the methods and DNA pellets were resuspended in 50  $\mu$ l of TE and RNAase (20  $\mu$ gml<sup>-1</sup>).

A simple test for the integrity of the *RAD54*-GFP cassette in pWDH445 was to utilise the modular nature of the plasmid to liberate the cassette by restriction endonuclease digestion (see experimental procedure 1.2.5.4.1). This module is released by double digestion of pWDH445 with *Bam*H I and *Asc* I restriction enzymes to give a 3177 bp fragment from the normal plasmid. pWDH445, plasmid prepared from 2 independent bright transformants, and plasmid isolated from a normal transformant were digested with *Bam*H I and *Asc* I, and the resulting DNA fragments separated by agarose gel electrophoresis. **Figure 2** shows the bands produced after ethidium bromide staining for the above restriction digests. All 4 pWDH445 reactions produce a band of ~3.2 kb suggesting that the *HO-RAD54*promoter-*yEGFP* cassette is unchanged from the normal pWDH445 (lanes 1 and 3) to those isolated from bright variants (lanes 2 and 4). However, the band representing the remainder of the plasmid is larger in one of the variants (lane 2) and smaller in the other (lane 4), whilst it remains constant between the DNA used in transformation and that isolated from a transformant showing normal brightness levels.

#### **1.3.1.4 - Yeast re-transformed with rearranged plasmid are G418 sensitive**

Plasmid DNA isolated from yeast and then prepared from *E. coli* after amplification was used to re-transform FF18984 yeast with normal and rearranged pWDH445, using the lithium acetate / PEG / SS-DNA protocol described in method 1.2.5.2. Transformants were selected by their ability to grow on medium deficient in uracil, since this was apparently unaffected by the mutations / rearrangements in the variants. The auxotrophic markers of transformants were checked as before and corresponded to those of the FF18984 background, with the exception of the ability to grow without uracil, conferred by pWDH445. Once more, resistance to G418 was tested by transferal of transformants to YPD plates containing  $200 \mu\text{gml}^{-1}$  G418. **Figure 3** shows photographs taken of re-transformed FF18984 incubated on YPD plates containing G418 and SD plates lacking uracil. The uracil-deficient plates are represented in the right-hand column and as expected demonstrate growth in all cases. However, the G418 plates (left-hand column) reveal that cells carrying the control pWDH445 and the normal pWDH445 isolated from yeast are G418 resistant as expected, but cells bearing either of the 2 anomalous plasmids are sensitive to  $200 \mu\text{gml}^{-1}$  G418. This suggests that the mutations or rearrangements are stable in that they were not reverted by the mutational effects sometimes associated with the transformation procedure.

#### **1.3.1.5 - Re-transformed G418-sensitive yeast still give brighter signal outputs**

FF18984 cells re-transformed with the altered reporter plasmids were tested for their ability to induce the reporter in response to 0.005% MMS, compared with cells carrying the normal pWDH445. After 15 hours incubation with MMS, fluorescence and scatter measurements were made and brightness values determined. **Figure 4** depicts the brightness signals from both cells bearing normal pWDH445 (darker bars) and cells conveying the altered plasmids (lighter bars). As previously, the lighter bars for the transformed cells are significantly larger than the corresponding darker bars. The modified plasmids gave rise to a 3.3 fold increase in the untreated brightness signal and a 4.6 fold increase in the MMS-induced signal, compared with cells bearing the unchanged pWDH445. Overall these increases had little effect on the induction ratio, producing only a slight decrease from 5.5 to 5.2 in

cells bearing modified plasmid. However, the increase in brightness signal with the re-transformants was not as great as for the original variants isolated.

### **1.3.2 Restriction analysis of pWDH445 and its brighter derivatives**

It was hypothesised that there was a link between the enhancement of brightness signal and the loss of G418 resistance, which suggested a change in or the loss of the *kanMX* module. Investigation of this required the use of diagnostic restriction digestions.

A restriction map of pWDH445 defined in this study is shown in **Figure 5**.

#### **1.3.2.1 - *Xba* I digestion revealed size differences in the rearranged plasmids**

The *Bam*H I and *Asc* I double digestion employed previously to confirm the presence of the intact *HO-RAD54* promoter-*yEGFP* module also revealed that one rearranged plasmid was larger than pWDH445 whilst the other was smaller. *Xba* I digestion was used to confirm the size changes in the rearranged plasmids after fractionation of the DNA fragments by agarose gel electrophoresis. From the map, *Xba* I digestion to completion was expected to yield 2 bands in a doublet formation at ~6 kb, for pWDH445 and plasmid isolated from cells exhibiting normal brightness. In **Figure 6**, lanes 1 and 3 represent the fragments created by *Xba* I digestion of pWDH445 and plasmid from cells of normal brightness, separated on a 1% agarose gel and labelled with ethidium bromide. Both lanes show identical bands in a doublet formation at an approximate size of 6 kb (lane 5 contains 500 bp ladder molecular weight marker DNA, with the largest fragment at 5 kb). Lane 4 represents the *Xba* I fragments for the rearranged plasmid that appeared to be smaller in the *Bam*H I – *Asc* I double digestion. The smaller size is also reflected in the replacement of the larger band from the doublet (~6 kb) with a band at ~4.4 kb, giving a vector size of ~10.4 kb as opposed to the 12 kb of pWDH445. Lane 2 shows the *Xba* I fragments for the other rearranged plasmid that appeared to be larger than pWDH445 in the *Bam*H I – *Asc* I digestion. As with the smaller rearranged plasmid, the larger band from the 6kb doublet is lost, but replaced not only with a band of ~4.4 kb, but also 2 further new bands at ~3.2 and 3.0 kb. This would suggest a vector size of ~16.6 kb, an increase of ~4.6 kb compared with pWDH445. This suggests that the brightness increase in cells carrying the rearranged plasmids was not purely a vector size-related phenomenon

(commonly there is an inverse relationship between size and copy number). It is also suggested that both rearrangements occur in the same half of the vector since the larger of the ~6 kb bands is lost in each case. The larger band represents the half of the plasmid bearing *Amp<sup>r</sup>*, *kanMX3*, and *yEGFP*. This further corroborated the suggestion from the *Bam*H I – *Asc* I digestion, that the *HO-RAD54* promoter-*yEGFP* module was intact after rearrangement.

#### 1.3.2.2 - *Sca* I digestion further limited the region of rearrangement

pWDH445 contains four recognition sites for the *Sca* I restriction enzyme and hence *Sca* I digestion should result in the production of four fragments. The four fragments should be represented by bands at approximately 4.7, 4.1, 1.9, and 1.3 kb after electrophoretic separation. **Figure 7** shows a picture of the ethidium bromide stained 1% agarose gel used to fractionate the *Sca* I fragments from pWDH445 (lane 2), the larger rearranged plasmid (lane 3), re-isolated pWDH445 (lane 4), and the smaller rearranged plasmid (lane 5). Lanes 2 and 4 show the same patterns of bands, with a doublet between 4 and 5 kb (representing the 4.7 and 4.1 kb fragments) and single bands at ~1.8 kb and 1.3 kb. Neither of the rearranged plasmids (lanes 3 and 5) exhibit the 1.3 kb *Sca* I band, the production of which requires the *Sca* I recognition site within *kanMX3* to be intact. The ~1.8 and 4.1 kb bands are present in all 4 of the plasmids digested, implying that no alteration had occurred in the region containing *HO*, *URA3*, the 2 micron origin, or *Amp<sup>r</sup>*. Hence, the portion of the vector in which the rearrangements could have occurred was further limited to the region between the unique *Asc* I recognition site and the *Sca* I recognition site in *Amp<sup>r</sup>*, including the entire *kanMX3* module.

#### 1.3.2.3 - The *Pst* I cleavage site within the *kanMX3* module is lost in the rearranged plasmids

Once the region of the plasmid incorporating the rearrangements was established, it was necessary to test restriction enzymes with cleavage sites in this section. The *Pst* I cleavage site occurs 7 times in pWDH445, though only 6 bands were expected to be detectable on an ethidium bromide stained 1% agarose mini-gel after electrophoretic separation of the *Pst* I fragments. The seventh fragment was too small to be detected under these conditions. Approximate sizes for the 6 bands were 4.2, 2.5, 2.2 or 1.6, 1.4, 1.3, and 0.9 kb. The fragment expected to be detected at 2.2



or 1.6 kb should result from cleavage at the sites within *HO* and *URA3*, but the size is dependent upon the orientation of the *URA3* gene.

**Figure 8** shows a picture of the ethidium bromide stained gel in which the fragments from *Pst* I digested plasmid were separated. pWDH445 and re-isolated pWDH445 were loaded into lanes 2 and 4, respectively, whilst the larger and smaller rearranged plasmids were loaded into lanes 3 and 5, respectively. All four plasmids exhibit a 1.6 kb band suggesting that the *URA3* gene is transcribed in the opposite direction to the *HO-RAD54* promoter-*yEGFP-kanMX3* cassette. The 2 bands involving the *Pst* I cleavage site in the *kanMX3* module are the 2.5 and 0.9 kb fragments. Only lanes 2 and 4, containing digested pWDH445 and digested re-isolated pWDH445, produced bands at 2.5 and 0.9 kb (the 0.9 kb band is not visible in **Figure 8**) whilst these were absent for both rearranged plasmids (lanes 3 and 5). Loss of the 2.5 kb and 0.9 kb bands from both of the rearranged plasmids suggests that a rearrangement had occurred within *kanMX*, causing loss of the *kanMX* *Pst* I cleavage site. *Pst* I digestion of both of the rearranged plasmids produced a novel 1.5 kb band not present in the pWDH445 digestions. If only the *Pst* I cleavage site in *kanMX3* had been altered in such a way as to prevent its cleavage, the appearance of a ~3.5 kb band would be expected (combination of the 2.5 and 0.9 kb fragments). However, recombination between the direct repeat elements flanking the *Kan<sup>r</sup>* gene in the *kanMX3* module could result in the loss of a 2 kb portion of the 3.5 kb fragment. This may account for the 1.5 kb band detected in lanes 3 and 5.

#### 1.3.2.4 - Two out of three *Pvu* I cleavage sites were lost in both rearranged plasmids

There are four cleavage sites for the *Pvu* I restriction endonuclease in pWDH445, 3 of which lie within the *kanMX3* module. The four fragments expected were of the sizes ~9, 1.2, 1.1, and 0.7 kb. The four plasmids were digested with *Pvu* I and the resulting fragments were fractionated on a 1% agarose gel by electrophoresis. The digested plasmids were loaded onto the gel with stock pWDH445 in lane 1, the larger rearranged plasmid in lane 2, isolated pWDH445 in lane 3, and the smaller rearranged plasmid in lane 4. **Figure 9** shows a picture of the ethidium bromide stained *Pvu* I fragments. Both lanes 1 and 3 exhibit all four of the expected bands whilst lanes 2 and 4 reveal different band patterns. The smaller rearranged plasmid in

lane 4 showed the large band, representing the bulk of the plasmid, and a band at 1.1 kb that all four plasmids exhibit. The 1.1 kb band results from cleavage by *Pvu* I in the *Amp<sup>r</sup>* and *Kan<sup>r</sup>* genes, suggesting that the *Pvu* I cleavage site at the 5' end of *kanMX3* was intact. However, neither rearranged plasmid shows bands representing the 1.2 and 0.7 kb *kanMX3* internal fragments. This suggests that a loss of DNA within the *kanMX3* module has occurred. The larger rearranged plasmid also showed 2 novel bands at 1 kb and ~3.5 kb.

#### **1.3.2.5 - *Sac* I does not liberate ~2.1 kb of *kanMX3* from the rearranged plasmids**

There are 2 *Sac* I cleavage sites in pWDH445, one in each of the direct repeat sequences flanking *Kan<sup>r</sup>* in *kanMX3*. Thus, 2 bands representing 2 fragments of ~2.1 kb and ~10 kb should be produced by *Sac* I digestion of pWDH445. The 2.1 kb fragment is released from *kanMX3* whilst the larger fragment represents the remainder of the plasmid. The four plasmids were digested with *Sac* I restriction endonuclease and the resulting fragments were separated electrophoretically in a 1% agarose gel. **Figure 10** is a picture of the ethidium bromide stained gel with pWDH445 in lane 1, the larger rearranged plasmid in lane 2, isolated pWDH445 in lane 3, and the smaller rearranged plasmid in lane 4. Only lanes 1 and 3 exhibit the expected 2.1 kb fragment released from *kanMX3*, whilst lanes 2 and 4 both show only a single band representing uncut plasmid. In conjunction with the other evidence discussed above, this suggests that both rearranged plasmids have lost a portion of *kanMX3* between and including the *Sac* I cleavage sites in the direct repeat sequences.

#### **1.3.3 Enzymic removal of *kanMX3* from pWDH445**

The nature of the investigations described above is such that a considerable amount of time could have been consumed in attempting to precisely define the rearrangement(s). Since a significant rearrangement was defined within the *kanMX3* module, it was decided to re-create this modification by enzymically excising *kanMX3*. This would provide a means to test the hypothesis that the enhancement of brightness from cells bearing the rearranged plasmids was due to the loss of part of the *kanMX3* module.

### 1.3.3.1 – *Sac* I digestion of pWDH445 followed by re-ligation

Stock pWDH445 was digested with *Sac* I to completion, in order to remove a ~2.1 kb fragment from the *kanMX3* module. A 3 µl aliquot of *Sac* I-digested pWDH445 was loaded onto a 1% agarose gel and the fragments separated by electrophoresis in order to check the success of the digestion (data not shown). After heat inactivation of the *Sac* I enzyme by incubation at 65°C for 20 minutes, digested pWDH445 was then re-ligated. Ligations were performed at 3 different concentrations of digested plasmid DNA (undiluted, 1 in 10 fold dilution, and 1 in 100 fold dilution) by incubation with T4 DNA ligase at 16°C overnight (method 1.2.5.4.2). Dilutions of digested DNA were used in order to promote intramolecular ligation events. A 10 µl aliquot of each ligation mix was used to transform *E. coli* and transformants were selected by overnight incubation at 37°C on LB plates containing ampicillin. Ampicillin resistant transformants were replica-plated onto LB plates containing kanamycin. After overnight incubation on kanamycin plates, very few colonies were able to grow.

10 ampicillin resistant colonies were selected, including one transformant that was also kanamycin resistant, and incubated with shaking in LB broth containing ampicillin at 37°C overnight. Plasmid preparations were made from these cultures using the method described in method 1.2.5.3.2 and the isolated DNA digested with *Xba* I along with normal pWDH445. Fragments were separated by electrophoresis in a 1% agarose gel and the ethidium bromide stained gel is shown in **Figure 11**. Lanes 1 and 13 show the bands representing the *Xba* I fragments from pWDH445, though overloading makes the 6 kb fragment difficult to distinguish. Lane 7 contained a 500 bp ladder molecular weight marker with the largest band at 5 kb, demonstrating that the bands in lanes 1 and 13 were ~6 kb. Lane 10 contained DNA from the kanamycin resistant colony and reveals the same 6 kb band as the controls in lanes 1 and 13. The remainder of the lanes contained DNA from kanamycin sensitive colonies and all exhibit a band at ~6 kb, but also a second band at ~4.5 kb. This is the same banding pattern as that seen for *Xba* I-digestion of the smaller rearranged plasmid (see lane 4, **Figure 6**).

### 1.3.3.2 - The new plasmid is brighter than pWDH445 in yeast

FF18984 cells were transformed with DNA from preparations of the new plasmid using the method described in 1.2.5.2.1. Transformants were selected for by incubation on SD plates lacking uracil and the auxotrophic markers were checked. 10 transformants were picked for assessment of brightness in response to 0.005% MMS. Incubation with MMS lasted 15 hours before measurement of fluorescence and scatter. The average brightness values determined for cells bearing the new plasmid (red bars) are compared with those from cells carrying pWDH445 (blue bars) in **Figure 12**. The brightness values for the cells carrying the plasmid with part of *kanMX3* enzymatically excised (**Figure 12**) were comparable with those of cells bearing the spontaneously modified plasmid isolated from yeast. Untreated cells bearing the new plasmid were found to be 4 fold brighter than those conveying pWDH445, whilst the brightness increased 5 fold for cells treated with MMS. However, the induction ratio was reduced in the brighter cells due to the increase in the constitutive level of brightness.

## 1.4 SUMMARY

1. Transformation of FF18984 with pWDH445 generated some transformants with a 12 fold brighter constitutive signal and a 9 fold brighter MMS-induced signal than typical pWDH445-bearing FF18984 cells. The induction ratio was halved in the brighter cells.
2. Bright transformants were unable to grow on medium containing G418, suggesting an alteration within the *kanMX3* module of pWDH445.
3. Double restriction of plasmids isolated from the brighter transformants with *BamH* I and *Asc* I produced the same size bands as pWDH445, suggesting that the *HO-RAD54* promoter-*yEGFP* module was unchanged. However, one rearranged plasmid was larger than pWDH445 whilst another was smaller.
4. Yeast cells (FF18984) re-transformed with rearranged plasmid were sensitive to G418, confirming that the change in brightness was due to the plasmid change, as opposed to a chromosomal mutation.
5. Untreated and MMS-induced re-transformed yeast cells were 3.3 fold and 4.6 fold brighter than pWDH445-carrying cells, respectively. However, there was little change in induction ratio between cells conveying pWDH445 or rearranged plasmid.

6. Digestion with *Xba* I corroborated the finding from *Bam*H I-*Asc* I double digestion that one plasmid was larger and one smaller than pWDH445. The fragment altered by the rearrangements carries *Amp*<sup>r</sup>, *kanMX3*, and *yEGFP*.
7. *Sca* I digestion suggested that no rearrangement had occurred in the region bearing *HO*, *URA3*, the 2 micron origin, and part of *Amp*<sup>r</sup>. In conjunction with the findings from the *Bam*H I-*Asc* I and *Xba* I digestions, rearrangement must have occurred between the unique *Asc* I cleavage site and the *Sca* I cleavage site in *Amp*<sup>r</sup>, in a region incorporating the entire *kanMX3* module.
8. The 2 bands formed by *Pst* I digestion if the *Pst* I site in *kanMX3* is intact are not present for either of the rearranged plasmids.
9. 2 of 3 *Pvu* I cleavage sites in *kanMX3* are not present in the rearranged plasmids suggesting loss of DNA within the *kanMX3* module.
10. *Sac* I does not release the expected ~2.1 kb *kanMX3* fragment from the rearranged plasmids.
11. A ~2.1 kb fragment of *kanMX3* was cleaved from pWDH445 using *Sac* I before religation of the *Sac* I sticky ends. Yeast cells transformed with the new plasmid were as bright as the yeast cells bearing spontaneously rearranged plasmids.

## **1.5 DISCUSSION**

Rearrangements of pWDH445 (to form recombinant vectors according to the first aspect of the invention) occurred, probably in a transformation-dependent manner given that transformation is known to be a mutagenic process, generating brighter than normal transformants. These bright transformants were found to be sensitive to growth on medium containing 200 µgml<sup>-1</sup> geneticin, despite pWDH445 bearing a *kanMX* module incorporating the gene encoding the aminoglycoside phosphotransferase that confers resistance to geneticin on yeast cells. This suggested that mutation or loss of the *kanMX* module had occurred, leaving the cells sensitive to geneticin. It was anticipated that *kanMX* had probably been lost from the plasmid, since a deletion event would reduce the size of the plasmid, increasing its stability and hence copy number.

However, after isolation of plasmid DNA from the bright transformants and digestion to release the *HO-RAD54*promoter-*yEGFP* cassette, it was clear from the

bands representing the DNA fragments separated by gel electrophoresis that whilst one bright transformant carried a smaller reporter plasmid, another conveyed a larger derivative of pWDH445. This ruled out the hypothesis that the enhanced reporter output was a plasmid size-related phenomenon and thus, demanded more detailed investigation. The outcome of further restriction analysis of the rearranged plasmids revealed that *kanMX* was lost in both the smaller and larger plasmids, and the larger plasmid gained several kb of DNA.

The smaller plasmid was probably generated by recombination between the direct repeats that flank the *kan<sup>r</sup>* gene and promoter sequences within the *kanMX* module. The *kanMX* fragment released by *Sac* I digestion is ~2.1 kb which is approximately the size of fragment lost by the small plasmid.

It has been previously shown that bacterial sequences borne by shuttle vectors can be toxic to heterologous host organisms and that such sequences unnecessary for maintenance in *S. cerevisiae* can be automatically eliminated by recombination events. Since all pWDH445 transformants were primarily selected for their ability to grow on SD medium lacking uracil, the *kanMX* module was unnecessary for plasmid maintenance.

Seemingly the enhancement of reporter brightness is not related to a reduction in size and hence increase in copy number, since one of the rearranged plasmids was shown to have increased in size. This suggests that the constitutive expression of the aminoglycoside phosphotransferase driven by the promoter from the translation elongation factor 1 $\alpha$  (*AgTEF*) from the filamentous fungus *Ashbya gossipyii*, affects the expression of yEGFP from the *RAD54* promoter in some way. The *kanMX* module is immediately downstream of the *HO-RAD54*promoter-yEGFP cassette with only a direct repeat sequence (465 bp) separating yEGFP from *AgTEF*. While we do not wish to be bound by any hypothesis, it is conceivable that the omnipresent (due to the constitutive expression) transcription complex for the aminoglycoside phosphotransferase interferes with termination of yEGFP transcription, resulting in imperfect transcripts and hence nascent yEGFP polypeptide that is incapable of legitimate folding to produce mature GFP. Loss of *kanMX* would remove the

interference and permit a greater proportion of yEGFP to fold correctly and hence fluoresce, in both constitutive and induced reporter states.

Interference of efficient transcription of *yEGFP* might result from the conformation of the plasmid. The size and sequence of pWDH445 bearing *kanMX* might be such that the plasmid assumes a conformation restricting access of components of the transcriptional complex to the *RAD54* promoter. However, loss of *kanMX* regardless of the mechanism could result in alteration of the conformation, due to the change in size and sequence, leading to greater accessibility of the *RAD54* promoter. In this case, it must be that pWDH445 is of the critical size allowing it to assume the restrictive conformation, and hence any change in size, either increase or reduction, alters the conformation sufficiently.

An alternative explanation for the enhanced brightness in the absence of *kanMX* is the greater availability of general transcription factors. Since the aminoglycoside phosphotransferase encoded within *kanMX* is constitutively expressed, it must have a constant requirement for general transcription factors. Thus, the availability of such factors for transcription from the *RAD54* promoter is reduced in the presence of *kanMX*, and removal of this module eliminates the constraints on the reporter cassette. This is most likely only a “local” deficiency in the volume occupied by the plasmid, and might reflect a higher affinity for the transcription factors in the *AgTEF* promoter.

Finally, *kanMX* related interference with reporter expression might occur post-transcriptionally. Both yEGFP and the aminoglycoside phosphotransferase are heterologous proteins since GFP originates from *Aequorea victoria* and *Kan<sup>r</sup>* from the *E. coli* transposon *Tn903*. It is possible that the 2 foreign proteins interact, preventing efficient protein folding and maturation of yEGFP. Loss of *kanMX* would result in loss of the illegitimate interaction, increasing the proportion of fluorescent yEGFP.

The mechanism by which *kanMX* is lost and reporter output is enhanced is essentially irrelevant to the commercial development of the reporter (as long as the response remains predictable), since a brighter reporter is obviously beneficial. The *kanMX* module was employed as a convenient selectable marker for research

purposes, but stringent rules governing the release of genes conferring antibiotic resistance would require its displacement for commercialisation.

If the *kanMX* effect seen with pWDH445 is purely a plasmid-related phenomenon then the reporter cassette could be re-cloned into a different plasmid backbone, in order to determine if the effect is specific to pWDH445. Replacement of *kanMX* with a different gene conferring antibiotic resistance, such as hygromycin B (*hph*; *hphMX*), nourseothricin (*nat*; *natMX*), and bialaphos (*pat*; *patMX*), would resolve whether the effect is specific to *kanMX* on pWDH445. An intergenic region could be inserted between *yEGFP* and *kanMX* on pWDH445 to determine whether the effect is caused by the proximity of the *AgTEF* promoter of *kanMX* to the termination codon of *yEGFP*. Assessment of these new vectors would be by constitutive and induced brightness values when borne by the FF18984 strain. Bright transformants have been isolated from other yeast strains though plasmid DNA has not been isolated and assessed in the same way as from bright FF18984 cells. However, it is anticipated that similar rearrangements would be detected in plasmid isolated from alternative strains, which would discount a strain-specific effect.



## **EXAMPLE 2**

A yeast DNA repair reporter assay termed the GreenScreen<sup>®</sup> assay (GSA) is described. This assay represents a preferred method of detecting for the presence of an agent that causes DNA damage according to the third aspect of the invention.

We describe the simple, robust assay protocol, the development and a validation study; results of which indicate that compounds giving positive responses have a high likelihood (96%) of corresponding positive responses in one or more of the regulatory tests (Ames, Micronucleus and Mouse Lymphoma Tests or carcinogenicity bioassays). The false positive rate is extremely low. The endpoint of the test reflects the typically eukaryotic chromosomes and DNA metabolising enzymes of *Saccharomyces cerevisiae*. The capacity for metabolic activation (MA) is limited compared to the mammalian liver or its extracts, but the assay does detect a subset of compounds that would require MA in existing genotoxicity tests. The GSA detects a different spectrum of compounds to bacterial genotoxicity assays and thus, together with an *in silico* Structure Activity Relationship (SAR) screen, and possibly a high throughput bacterial screen, would provide an effective preview of the regulatory battery of genotoxicity tests.

### **2.1 Introduction**

The introduction of high-throughput bacterial screens such as the SOS *umu* and SOS *lux* tests have demonstrated that it is possible to apply genotoxicity testing to the small quantities of test chemical available at the early stages of the drug discovery process. This type of assay may also have an important role in such diverse disciplines as the environmental monitoring and food industries and military field-testing. Although these bacterial assays have proved their effectiveness, they have some disadvantages; especially because they use non-eukaryotic cells and hence will not detect genotoxins that interact with eukaryotic-specific targets.

The inventors have previously reported the construction and preliminary validation of a yeast-based genotoxicity test system, *RAD54*-GFP (see WO 98/44149). Induction of the *RAD54* promoter results in the production of the extremely stable Green Fluorescent Protein (GFP), which is fluorescent in the green spectrum when illuminated by blue light. It appears that any agent able to cause

mutation in yeast will lead to *RAD54* induction. The specificity to DNA damage has been confirmed by studying the global transcriptional response to DNA-damaging agents using DNA microarrays. It was found that *RAD54* did not respond to other non-genotoxic stresses, such as heat shock, oxidative stress, reductive stress, osmotic shock or amino acid starvation.

*RAD54* encodes a structural element of the homologous recombinational (HR) repair pathway, but responds to a broad spectrum of genotoxins, suggesting that the yeast DNA damage sensing pathways activate the HR pathway as a default for failure of the other repair pathways. This is supported by several lines of genetic evidence. For example the loss of the non-homologous end-joining (NHEJ) pathway in yeast is undetectable unless the *RAD52* (HR) pathway is also ablated. Furthermore, it has been demonstrated that induction of *RAD54* by methyl methanesulfonate (MMS) is insensitive to mutations in the 'classical' DNA-damage sensing pathway controlled by the *RAD9* and *DDC1* checkpoints. This suggests that there are additional DNA damage sensing pathways leading to *RAD54* activation and that this may account for its broad response profile. The HR DNA-repair process is well characterised and is apparently highly conserved within all eukaryotic organisms. Expression of the human homologue *hRAD54* in yeast largely suppresses the phenotypes resulting from deletion of the native copy. Increased expression of *RAD54* in yeast as a result of exposure to genotoxins is therefore likely to be predictive of the increased DNA repair activity in mammalian cells.

Here we describe a protocol suited to the use of robotic liquid handling systems and present simple data handling protocols that provide clear graphical output. We report the results of an assay validation program in which 102 compounds with diverse genotoxic potential have been tested.

## **2.2 Materials and Methods**

### **2.2.1 Strains & Plasmids.**

The yeast strains, plasmids and growth media (F1) used in this study have been described previously (Walmsley *et al.*, 1997) and the use of Green Fluorescent Protein (GFP) as a reporter for the DNA damage-induced gene *RAD54* from *Saccharomyces cerevisiae*, has also been described (Billinton, *et al.*, 1998). The *Saccharomyces*

*cerevisiae* strain FF18984 (*MATa*, *leu2-3,112* *ura3-52* *lys2-1* *his7-1*) was obtained from Francis Fabre (French Atomic Energy Commission (CEA), Fontenay-aux-Roses, France).

The reporter strain (GenT01) is FF18984 containing a replicative plasmid containing the entire upstream non-coding DNA sequence of the *RAD54* gene fused to the yeast-enhanced *Aequorea victoria* GFP gene. The control strain (GenC01) is FF18984 containing an identical plasmid except that 2 base pairs have been removed at the start of the GFP gene, such that no GFP is made.

### 2.2.2 Microplate preparation.

Assays were carried out in 96 well, black, clear-bottomed microplates (Matrix ScreenMates, Cat. No. 4929, Apogent Discoveries, USA). A number of alternative microplates were assessed, though the variable background absorbance and fluorescence both within and between plates from individual manufacturers were generally unacceptable, leading to the conclusion that only Matrix or Corning (BV, Netherlands: Cat. No. 3651) plates were appropriate for this assay. The assays were performed using a liquid handling robot (MicroLabS single probe, Hamilton GB Ltd., Birmingham. UK) in a protocol designed to set up 4 compounds per test on a single 96 well microplate in 30 minutes. Results for a subset of compounds have been reproduced using a Genesis 8-probe robot (Tecan UK Ltd. Theale. UK), which can set up a similar microplate in less than 5 minutes. Microplates can also be filled rapidly and effectively using a multi-channel pipette.

A microplate version of the assay has been previously reported (Afanassiev *et al.*, 2000), but different microplate layouts and controls have been used for this study, so more detail is presented here. The following standard protocol was followed. A 1 mM stock of the test chemical was prepared in 2% v/v aqueous DMSO, and used to make 2 identical dilution series across the microplate and a 'control' (see below). To achieve this, 150 microlitres of the test chemical solution were put into 2 microplate wells. Each sample was serially diluted by transferring 75 microlitres into 75 microlitres of 2% DMSO, mixing, and then taking 75 microlitres out and into the next well. This produced 9 serial dilutions of 75 microlitres each.

Controls were added as follows:

- a. Compound alone, to provide information on compound absorbance/fluorescence
- b. Yeast cultures diluted with 2% DMSO alone, to give a measure of maximum proliferative potential
- c. MMS as a genotoxicity control: 'high' = 0.00125% v/v, 'low' = 0.0001875% v/v
- d. Methanol as a cytotoxicity control: 'high' = 3.5% v/v, 'low' = 1.5% v/v.
- e. Growth medium alone, to confirm sterility/lack of contamination

Stationary phase cultures of GenT01 and GenC01 were diluted to an optical density ( $OD_{600nm}$ ) = 0.2 in double strength F1 medium (Billinton *et al.*, 1998). 75 microlitres of the yeast suspension were added to each well of the diluted chemical: GenT01 to one series and GenC01 to the second, and to appropriate standards and controls. After the plates were filled, they were sealed using either a gas permeable membrane (Breath-easy, Diversified Biotech, USA) or a plastic lid, and then incubated without shaking, overnight at 25°C.

### **2.2.3 Compounds chosen for the study.**

In order to compile a list of chemicals unbiased by the inventors' laboratory, a number of pharmaceutical companies and contract research organisations were asked to suggest compounds for which results would be of interest in assessing a new test. All compounds were sourced at analytical purity where available (Sigma, Aldrich, Fluka, BDH, Avocado).

### **2.2.4 Data collection and handling.**

Following overnight incubation, fluorescence and absorbance data were collected from the microplates. Two different microplate readers which combine fluorescence and absorbance functionality have been used, and comparable data were obtained. These were a Tecan Ultra-384 (Tecan UK Ltd.): excitation 485 nm / emission 535 nm with an additional dichroic mirror (reflectance 320 nm – 500 nm, transmission 520 nm – 800 nm), and a BMG PolarStar (BMG Labtechnologies, Germany): excitation 485 nm / emission 520 nm. Absorbance was measured through a 620 nm filter in both instruments. The data were transported into a Microsoft Excel spreadsheet, and converted to graphical data (see typical data in Figure 18). Data

processing is minimal: absorbance data give an indication of reduction in proliferative potential and these data were normalised to the untreated control (=100% growth). Fluorescence data were divided by absorbance data to give 'brightness units', the measure of average GFP induction per cell. These data were normalised to the untreated control (=1). In this way, one can distinguish between a small number of highly fluorescent cells and a large number of weakly fluorescent cells. In order to correct for induced cellular autofluorescence and intrinsic compound fluorescence, the brightness values for the GenC01 strain were subtracted from those of GenT01. This makes visual assessment of the data more reliable. All the data were checked with and without this correction, and the decision (see below), on whether or not a compound was classified as being genotoxic, was not affected.

#### **2.2.5 Decision thresholds.**

It is useful to have clear definitions of positive and negative results from routine assays and such definitions have been derived, taking into account the maximum noise in the system and data from chemicals where there is a clear consensus on genotoxicity and mechanism of action. Naturally it is also possible for users to inspect the numerical and graphical data and draw their own conclusions. For example an upward trend in genotoxicity data that did not cross the threshold might still distinguish two compounds. The decision thresholds were set as follows:

The cytotoxicity threshold is set at 80 % of the cell density reached by the untreated control cells. This is greater than 3 times the standard deviation of the background. A positive cytotoxicity result (+) is concluded if 1 or 2 compound dilutions produce a final cell density lower than the 80% threshold. A strong positive cytotoxicity result positive (++) is concluded when either (i) three or more compound dilutions produce a final cell density lower than the 80% threshold or (ii) at least one compound dilution produces a final cell density lower than a 50% threshold. A negative result (-) is concluded when no compound dilutions produce a final cell density lower than the 80% threshold. The lowest effective concentration (LEC) is the lowest test compound concentration that produces a final cell density below the 80% threshold.

The compound absorbance control allows a warning to be generated if a test compound is significantly absorbing. If the ratio of the absorbance of the compound control well to a well filled with diluent alone is  $> 2$ , there is a risk of interference with interpretation. The cytotoxicity controls indicate that the yeast is behaving normally. The 'high' methanol standard should reduce the final cell density to below the 80% threshold, and should be a lower value than the 'low' standard.

The genotoxic threshold is set at a relative GFP induction of 1.3 (i.e. a 30% increase). This is greater than 3 times the standard deviation of the background. A positive genotoxicity result (+) is concluded if 1 or 2 compound dilutions produce a relative GFP induction greater than the 1.3 threshold. A strong positive genotoxicity result (++) is concluded if either (i) three or more compound dilutions produce a relative GFP induction greater than the 1.3 threshold or (ii) at least one compound dilution produces a relative GFP induction greater than a 1.6 threshold. A negative genotoxicity result (-) is concluded where no compound dilutions produce a relative GFP induction greater than the 1.3 threshold. The LEC is the lowest test compound concentration that produces a relative GFP induction greater than the 1.3 threshold. The genotoxic controls demonstrate that the strains are responding normally to DNA damage. The 'high' MMS standard must produce a fluorescence induction  $> 2$ , and be a greater value than the 'low' MMS standard. Anomalous brightness data is generated when the toxicity leads to a final cell density less than 30% that of the blank. Genotoxicity data is not calculated above this toxicity threshold. Compounds that tested negative for genotoxicity, were re-tested up to 10mM, or to the limit of solubility or cytotoxicity.

The compound fluorescence control allows a warning to be generated when a compound is highly auto-fluorescent. If the ratio of the fluorescence of the compound control well to a diluent filled well is  $> 5$ , there is a risk of interference with interpretation. In these cases (four in this study), fluorescence polarisation can be used to distinguish GFP from other sources of fluorescence (Knight *et al.*, 2000, 2002). Both the Tecan and BMG instruments have this facility. Occasionally, compounds though not fluorescent themselves, induce cellular auto-fluorescence. This is apparent from rising brightness in the control (GenC01) strain in the absence

of fluorescence from the chemical-only control. The routine subtraction of GenC01 from GenT01 data removes this interference from the data.

#### **2.2.6 Comparisons.**

Published results from other genotoxicity tests and cancer studies have been tabulated. They include Ames test, Mouse Lymphoma Assay (MLA), *in vitro* and *in vivo* cytogenetics (chromosome aberration assays) and rodent Micronucleus Test (MNT). The data have been collected both from the peer-reviewed literature and from freely available internet resources. These include CCRIS (Chemical Carcinogenesis Research Information System: <http://toxnet.nlm.nih.gov>), NTP (National Toxicology Program Reports: <http://ntp-server.niehs.nih.gov>), IARC (International Agency for Research on Cancer: <http://www.iarc.fr>), NIOHS (National Institute for Occupational Health and Safety: <http://www.cdc.gov/niosh/homepage.html>) and the USEPA (Environmental Protection Agency: <http://www.epa.gov/iris/search.htm>).

There are several compounds where there are conflicting data from different sources (recorded as +/-). However, where comparisons have been drawn these are recorded as positive (+). The only exception to this is where NTP has more recently suggested a definitive designation. It is not intended in this paper to reclassify chemicals on the basis of other test results and the authors apologise for any inadvertent gaps or confounding decisions.

#### **2.3 Results**

The results of the validation study incorporating 102 chemicals are shown in full in **Figure 20**. For completeness it includes some compounds tested at very high concentrations where positive genotoxicity results have been concluded from higher concentrations in other studies.

In comparison with the other test data, it is clear that the sensitivity of the test is high. 54 compounds were positive for genotoxicity in GSA, 50 of which had comparable data from other genotoxicity and cancer studies. Excluding the cancer results, 46/49 GSA positive compounds were positive in either Ames, MLA, MNT or chromosome aberration, giving a sensitivity of 94%. Considering just the mammalian

test data (i.e. excluding Ames data) 40 of 44 GSA positive compounds (91%) have positive data from MLA, MNT or chromosome aberration studies. However, if all data is included (i.e. mammalian, Ames and the 2 additional compounds with only positive cancer data, (3-amino-1,2,4-triazole and sulfamethoxazole), the sensitivity of the test to genetic damage increases to 96% (48/50). A second important statistic from the whole study is that there were only two compounds positive in GSA for which there was no corresponding positive data from other tests. These were tritoyl phosphate and cimetidine.

**Table 12:** *Suggested maximum tolerable solvent concentrations.*

Test Compound	CAS No.	Maximum Concentration (%)
		v/v)
Carbon tetrachloride	56-23-5	2
1-Chloro-2-propanol	127-00-4	2
Dimethylsulfoxide	67-68-5	5
Ethanol	64-17-5	2
Methanol	67-56-1	2
Tetrahydrofuran	109-99-9	2

Table 12 lists chemicals/solvents that are often used with test samples, to promote dissolution etc. All of these compounds are toxic at higher concentrations due to osmotic effects and/or effects on membranes, and interfere with data interpretation. If these chemicals are used to dissolve compound stock solutions, they should also be included in the diluent used to make the compound dilution series. The routine use of DMSO in this study revealed its predictable interference with certain compounds. For example, cisplatin was only positive when retested using water in place of 2% DMSO as the diluent. Interference of DMSO, a free radical scavenger, in the recombinogenic properties of several oxidising agents has previously been reported.



The data identified for Mouse Lymphoma Assays have a strong bias to positive results (only 7/56 were negative MLA). The comparative figures are as follows: Sensitivity 86% (25/29), Specificity 43% (3/7), Accuracy 78% (28/36).

In the group of chemicals with MNT data there was a similar bias with only 16 out of 59 compounds having negative data. Only 3 of these have both *in vivo* and *in vitro* study data. Comparisons include data for either or both tests. Sensitivity 76% (22/29), Specificity 56% (9/16), Accuracy 69% (31/45).

Comparative chromosome aberration test data was largely drawn from Ishidate *et al.*, (1988): Sensitivity 94% (16/17), Specificity 83% (5/6), Accuracy 91% (21/23).

The data identified for cancer studies also has a strong bias to positive results (only 17/69 studies were negative). The figures are as follows: Sensitivity 89% (32/36), Specificity 78% (14/18), Accuracy, 85% (46/54).

**Figure 21** contains the subset of data for which there are Ames data, and highlights the distinctively different endpoints for GSA and bacterial genotoxicity results. There are 93 compounds in this group. 18 compounds that are positive in GSA are negative for the Ames test (with or without S9), 17 of which are positive in animal carcinogenesis or mammalian cell tests. 6 of 31 GSA positives that are Ames positive, required metabolic activation for the Ames result. 16 Ames positive compounds were negative in GSA, of which 6 are only Ames positive with metabolic activation. The overall comparison with Ames data is as follows: Sensitivity 63% (31/49), Specificity 61% (28/46), Accuracy 62% (59/95). Results were also compared with the SOS *umuC* test, conceived as a screening preview of the Ames test (Reifferscheid and Heil, 1996). The comparison figures are as follows: Sensitivity 70% (19/27), Specificity 53% (9/17), Accuracy 64% (28/44).

## 2.4 Discussion

The induction of the yeast *RAD54* gene has been monitored using a promoter-GFP fusion. In a previous study (Afanassiev *et al.*, 2000), a small sample of chemicals (12) was selected to provide an indication of the range of DNA damaging

agents that could be detected. That study used a slightly different experimental protocol, though the results demonstrated a good coverage of DNA damaging agents as well as enzyme inhibitors including DNA polymerase (aphidicolin) and topoisomerase 1 (camptothecin) inhibitors.

In selecting compounds for this extended study it was decided that a very restricted list of compounds with expected results (unambiguous mutagens and non-mutagens) would not give a very broad definition of the endpoint. Broader selections of the 'obvious' in other studies have previously led to dispute about what the expected result should be. Thus the results presented here come from a collection of test compounds not selected by the authors. Naturally, it includes many obvious mutagens and non-mutagens; it also includes compounds with either conflicting genotoxicity data from other sources, or very little other data. The latter were clearly suggested to the authors to give a better idea of the test endpoint.

This broader study of 102 compounds (Figure 20) confirms that the *RAD54* gene is induced by a variety of DNA damage lesions caused by direct-acting agents. These include examples of base alteration (MMS, ethidium bromide), clastogenicity (chromosome breakage: bleomycin, catechol, 1,2-dimethylhydrazine, methyl viologen, methapyraline, phthalic acid bis(2-ethylhexyl) ester, phleomycin), and cross-linking (mitomycin C, cisplatin). Amongst the oxidising agents, cumene hydroperoxide is a notable negative. It is interesting that the photomutagen psoralen was a GSA positive as the test was only exposed to normal laboratory lighting. The reporter also responds to a variety of genotoxins that may not act directly on DNA. These include compounds that target topoisomerases (etoposide, ellipticine) as well as anti-mitotic, spindle targeting compounds (aneugens) such as colchicine and econazole. The latter results are interesting because they suggest that some aneugens can also lead indirectly to DNA damage. Importantly for a proposed screening test, only two positive results were unique to yeast, tritolyl phosphate and cimetidine. It would be interesting to discover the chemistry behind these results though it is intriguing that the latter (cyano-methyl-guanidine) is chemically related to methyl-nitro-nitrosoguanidine (MNNG), a well-known mutagen.

The group of other carcinogens not detectable by Ames (including safrole, urethane, aniline and thiourea) is interesting and here the GSA result confirms similar observations using the yeast DEL assay.

The spectrum of metabolic enzymes expressed by yeast is less complex than that of mammalian cells. There are, for example, the CYP (cytochrome P450) type enzymes (*ERG5* / *CYP61*; *DIT2* / *CYP56*; *P450<sub>14-DM</sub>* / *ERG11* / *CYP51*; the P450 oxido-reductase *NCP1* / *CPR1* and glutathione-S-transferases *GTT1* and *GTT2*. This proficiency is reflected by the identification of 6 compounds producing positive data with GSA that require S9 for an Ames positive result (Neutral Red, 2-amino-4-nitrophenol, proflavin hemisulfate, ethidium bromide, benzo[a]pyrene, 1-naphthylamine). S9 was not used in any of the GSA tests reported in this paper.

The metabolic capability of yeast contrasts to the enhanced activities in S9 required for the activation of most promutagens, and explains the most obvious group of compounds not detected by GSA: primary aromatic amines and aromatic amides. 9 such compounds were tested. Two were positive in GSA: 2-amino-4-nitrophenol and 1-naphthylamine. The others were negative in GSA and positive in other in vitro tests: 2-acetamidofluorene, 9-aminoacridine, 2-aminoanthracene, 4,4-oxydianiline, o-anisidine, aniline and 4-aminophenol. The last two of these are negative with and without S9 in Ames. The aromatic amine motif itself is recognised by SAR programmes so whilst missed by GSA, such compounds would be unlikely new drug candidates. It has been demonstrated that the metabolic competence of yeast strains could be enhanced by the expression of human cytochrome P450 genes. In preliminary experiments using plasmids from the Sengstag group in the yeast strains used in this study, the *RAD54-GFP* reporter has also increased its spectrum of response. A full assessment of these strains is underway (including an assessment of an S9 protocol) and these will be reported when complete.

To assess the relative compound requirement of the GSA when compared to bacterial screening tests, the Lowest Effective Concentrations (LEC) of a small sample of the validation chemical set were compared in Table 13.

This comparison indicates that the GSA is of comparable sensitivity to the SOS *umuC* chromotest and the SOS *lux* bioluminescence assay (based on a very limited dataset). Therefore the total compound requirement of the GSA is comparable to other commonly used bacterial short-term screens.

The GSA has a simple and fixed microplate protocol with clear decision-making thresholds. Together these properties were conceived to give a high throughput capability and to minimise the generation of conflicting test data. These are important requirements of any useful screen. The compounds tested in this screen provide sufficient data to reveal that a positive result with GSA provides a relevant warning of positive results in the regulatory battery of tests, and in the present form provides complementary data to bacterial tests – each providing significantly non-overlapping endpoints. It will be interesting to see how the definition of the endpoint develops as more data become available.

## 2.5 References

- Afanassiev, V., Sefton, M., Anantachaiyong, T., Barker, M.G., Walmsley, R.M., and Wölfl, S. (2000). Application of yeast cells transformed with GFP expression constructs containing the *RAD54* or *RNR2* promoter as a test for the genotoxic potential of chemical substances. *Mutat. Res.*, **464**, 297-308.
- Billinton, N., Barker, M.G., Michel, C.E., Knight, A.W., Heyer, W.-D., Goddard, N.J., Fielden, P.R. and Walmsley, R.M. (1998). Development of a green fluorescent protein reporter for a yeast genotoxicity biosensor. *Biosens. Bioelectron.*, **13**, 831-838.
- Walmsley, R.M., Billinton, N. and Heyer W.-D. (1997). Green fluorescent protein as a reporter for the DNA damage-induced gene *RAD54* in *Saccharomyces cerevisiae*. *Yeast*, **13**, 1535-1545.

**Table 13:** *A comparison of LEC data from GSA, umuC and lux assays.*

COMPOUND	S9	GSA (M)	SOS UMUC ASSAY (M)	SOS LUX ASSAY (M)
BENZO[A]PYRENE	+	$1.0 \times 10^{-4}$	NT	$7.9 \times 10^{-7}$
CISPLATIN	-	$6.2 \times 10^{-5}$	$9.0 \times 10^{-7}$	NT
HYDROGEN PEROXIDE	-	$2.0 \times 10^{-3}$	NT	$5.9 \times 10^{-5}$
METHYL METHANESULFONATE	-	$9.3 \times 10^{-6}$	$1.5 \times 10^{-4}$	$7.3 \times 10^{-5}$
MITOMYCIN C	-	$6.0 \times 10^{-4}$	$1.0 \times 10^{-8}$	$4.7 \times 10^{-8}$
MNNG	-	$1.7 \times 10^{-6}$	$6.0 \times 10^{-6}$	NT
NALIDIXIC ACID	-	$1.6 \times 10^{-6}$	$2.4 \times 10^{-6}$	$6.9 \times 10^{-7}$
SODIUM AZIDE	-	$1.0 \times 10^{-5}$	$5.4 \times 10^{-3}$	-

**KEY**

M = Molar concentration

NT = Not tested

MNNG = N-Methyl-N'-nitro-N-nitrosoguanidine

### **EXAMPLE 3**

This example provides a preferred protocol for carrying out the method of the invention using a plate assay procedure.

#### **1) PREPARING THE STARTER CULTURES.**

2 different strains are currently used per test: GenC01 (control strain) and GenT01 (the test strain). The cultures are inoculated and incubated in an orbital shaker at 200 rpm and 30 °C for three days (72 hours), after which the cells will be in 'stationary phase'. Culture flasks are then stored in a refrigerator (+ 4 °C) and can be used for tests over a period of up to seven days.

Each starter culture is prepared as follows :

Into a sterile 250 ml conical flask place -

10 ml sterile water  
10 ml 2X F1 media (See Appendix 1)  
1 x 250 µl colour coded aliquot of frozen cells

Seal flask with foam bung (taped to the flask) before incubating.

#### **2) PREPARING THE TEST COMPOUNDS.**

Standard solutions of the test compounds should be freshly prepared shortly before each test run. Unless a specific test concentration is required, the recommended top concentration of a test compound is 1 mM in 4 % DMSO v/v in water. It is necessary to ensure that the final concentration of test compound is made up in the same diluent as that supplied to the robot for serial dilutions. The diluent is 4 % DMSO v/v in water by default but can be substituted for water alone.

1 ml of test compound solution is required per 'test run' and there is sufficient capacity on a 96-well plate for 4 test runs. Therefore for each plate prepare 2 tubes each containing 1 ml of the respective test compound, label them, cover with black caps and leave in a fume cupboard until required. For reference record the details of the test compound, its concentration, dilution calculation and the diluent used.

#### **3) PREPARING THE CONTROL COMPOUNDS.**

The control compounds are prepared in 4% DMSO v/v in water to the following concentrations:

MMS High	=	0.002500 % v/v*
MMS Low	=	0.000375 % v/v*
Methanol High	=	7 % v/v*
Methanol Low	=	3 % v/v*

200  $\mu$ l of each control chemical is required per plate (this includes a volume required to allow safe automated pipetting distance). Hence for a set of 4 plates, make up at least 800  $\mu$ l of each control.

*\* By default all concentrations are halved when plated out, as a sample volume of 75  $\mu$ l is combined with 75  $\mu$ l of yeast culture producing a 50:50 dilution.*

*MMS and Methanol prepared from 100% stock solution with a diluent of 4 % DMSO / water (v/v).*

MMS:

5  $\mu$ l 100 % MMS + 995  $\mu$ l diluent = 0.5 % MMS Solution

Standard 1 (High): 20  $\mu$ l 0.5 % MMS + 3980  $\mu$ l diluent = 0.002500 % MMS

Standard 2 (Low): 450  $\mu$ l 0.0025 % MMS + 2550  $\mu$ l diluent = 0.000375 % MMS

Methanol:

Standard 1 (High): 280  $\mu$ l + 3720  $\mu$ l diluent = 7 % Methanol

Standard 2 (Low): 120  $\mu$ l + 3880  $\mu$ l diluent = 3 % Methanol

#### 4) PREPARING THE CELLS.

Measure the optical density (at 600 nm) of the 'stationary phase' GenT01 and GenC01 starter cultures using a 1 ml disposable cuvette with a 1 cm path length and water as a comparison blank. Due to the high density of the starter cultures, dilute 20 fold (950  $\mu$ l sterile water + 50  $\mu$ l cell culture) to enable measurement in the linear range. Calculate the OD of the undiluted cultures ( $Y$ ) using the equation below:

$$\text{OD of the starter culture (Y)} = \text{Measured OD} \times 20$$

Note: Once in cold storage (+ 4 °C) the cell density does not change significantly over the course of a week, and do not need to be re-measured before each day's use.

Prepare a fresh suspension of both GenT01 and GenC01 cells in 2X F1 media, at an initial OD of 0.2 units/ml. 4 ml of GenC01 and GenT01 cell suspensions are required per plate (3.3 ml plus dead volume). Therefore for 4 microplates the total volume of fresh cell suspension required is 16 ml for GenC01 and 16 ml for GenT01.

Use the following equation to calculate,  $V$ , the volume of starter culture required to prepare the new cell suspension:

$$V = \frac{0.2 \times \text{volume of fresh culture required (ml)}}{Y}$$

$Y$  = Optical density (O.D) of starter culture

Transfer  $V$  ml of stock cell culture into a sterile 5ml glass test tube containing 4mls of 2x F1 media.

Therefore for 4 microplates 16 ml of GenC01 is split into 4 x 4 ml aliquots in (**blue** capped) test tubes, and 16 ml of GenT01 is split into 4 x 4 ml aliquots (**red** capped) test tubes. For a lower number of plates scale down accordingly.

## 5) PREPARING ADDITIONAL TUBES AND SOLUTIONS.

Place 3.5 ml of 90 % ethanol in a single tube.

Place 3.5 ml of “media only” (1xF1) in a single tube.

Place approx. 900 ml of 10 % ‘Chlorox’ in a 1 L container as a disinfectant, and toxic chemical neutralising, solution into which caps and spent test tubes can be sterilised before washing, autoclaving and recycling.

## 6) SET-UP FOR THE PIPETTING ROBOT AND DECK LAYOUT.

### *Switching on and Software Set-up*

i. SWITCH ON the equipment in the following order.

- The fume hood.
- Computer.
- MVP (Diluent Selector Unit) at mains plug.
- The robot using the switch on right hand side.
- load up ‘Microlab Sampler Software’.

If the robot has been left filled with anything other than sterile water, purge the system with sterile water using a wash program before use. The following methods are available. To run use *File / Open* the click “Run Method”, “Start” and follow the on-screen instructions.

Wash Needle/System.Ame	Washes 10 ml of a solution connected to MVP position 1 into a waste container placed in the ‘Bleach wash’ position.
Wash Station/Tubing.Ame	Washes the needle and “wash station” and all associated pump tubing for cycles of 10 s, from the “clean wash” reservoir to waste.

ii. The control program reads in the settings for the chosen diluent, sample volume, yeast volume and the volume carried over in each serial dilution for each plate. The default values are as follows.



PLATE	DILUENT	SMPVOL	YEASTVOL	DILVOL1	DILVOL2	DILVOL3	DILVOL4
1,	1,	75,	75,	75,	75,	75,	75
2,	1,	75,	75,	75,	75,	75,	75
3,	1,	75,	75,	75,	75,	75,	75
4,	1,	75,	75,	75,	75,	75,	75

To alter the values or check the current configuration open the file  
C:/Program Files/Hamilton/Microlab Sampler/Methods/4COMPOUND.lay  
in Notepad. Make the alteration then select *File / Exit* and only when prompted  
with "The text in the file has changed. Do you want to save the changes?" select  
*Yes* to resave the file.

- iii. In the Microlab Sampler software select *File / Open* and open 4COMPOUND.lay
- iv. Select "Run Method".

#### *Loading the Robot Deck and Plating Out*

- i. Before commencing any method carefully clean the outside of the needle by hand with ethanol using a soft cloth.
- ii. Load the required number of Matrix micro-plates onto the robot deck in the positions as shown on the screen.
- iii. Load the GREEN RACK with the appropriate tubes containing yeast cultures, samples, standards, 90 % ethanol, plate blank and an addition one for waste volumes of the test compounds according to the loading sequence (see figure 21).
- iv. Place the appropriate diluent solutions in position to the right hand side of the deck with tube lines connected to the corresponding MVP selector positions. (Position 1 by default).
- v. Place the 'clean wash' tube in a container of sterilised water (approx. 300 ml per plate).
- vi. Ensure the 'dirty water' receptacle has sufficient capacity to collect the wash solution supplied.
- vii. Remove all tube caps and place in a suitable disinfectant / compound neutralising solution (i.e. 10 % Chlorox).
- viii. Select *START* in the 'Run Method' software.
- ix. The method will now run taking approximately 30 minutes to set up each plate according to the layout described in figure 22.
- x. Once the method is completed empty the remaining contents of the test tubes in the Green Rack into a sealable plastic bag containing a suitable absorbent

material. Seal, autoclave and dispose of the bag according to local regulations. Place the empty glass test tubes into the Chlorox solution for sterilisation and decontamination before subsequently washing and recycling.

#### **7) INCUBATING THE PLATES.**

Once the plate is complete cover with breathable membrane or plate lid and label clearly. Shake plate vigorously for 30 seconds on microtitre plate shaker (to fully mix the contents of each well) and then incubate at 25 °C, unshaken, for approximately 16 hours (or overnight).

#### **8) ROBOT MAINTENANCE**

Wash through the pipetting robot with sterile water. (See section 7 i for details). When leaving for long periods (i.e. > 2 days) leave the system filled with 70 % ethanol v/v in water. Approximately once a week wash through the system with a suitable disinfectant (i.e. dilute Chlorox solution) and then thoroughly with sterile water so that future user's solutions are not contaminated.

Clean the outside of the needle with a soft cloth soaked in ethanol.

Position the needle such that the needle tip is left immersed in sterile water contained in a test tube in the Green Rack, in position A1. (Once switched off the needle and robot arm can be moved manually taking care not to bend or damage the needle. Its position will re-set automatically when commencing a new method.)

#### **9) PLATE READING AND DATA ANALYSIS**

For plate reading and analysis refer to the protocol appropriate for the plate reader used. i.e. Tecan Ultra or BMG Polar Star.

#### **10) DISPOSAL**

When all results processing is completed, contain the used micro-plates within a sealed plastic bag containing a suitable absorbent material. Seal, autoclave and dispose of according to local regulations.



## **CLAIMS**

1. A recombinant vector comprising a recombinant DNA molecule comprising a regulatory element that activates gene expression in response to DNA damage operatively linked to a DNA sequence that encodes a light emitting reporter protein and a DNA vector characterised in that the vector comprises an origin of replication; at least one selectable maker; and when used to transform a cell, does not alter the sensitivity of the cell to geneticin.
2. A recombinant vector according to claim 1 comprising the vector of Figure 5 with a deletion, substitution or addition that disrupts the function of the KanMx3 gene.
3. A recombinant vector according to claim 1 comprising the vector of Figure 15 or a functional derivative thereof
4. A recombinant vector according to claim 1 wherein the recombinant DNA molecule comprising a yeast RAD54 regulatory element operatively linked to a DNA sequence that encodes a light emitting reporter protein
5. The recombinant vector according to any preceding claim wherein the light emitting reporter protein is Green Fluorescent Protein and light-emitting derivatives thereof.
6. The recombinant vector according to claim 5 wherein the recombinant DNA molecule has a DNA sequence encoding the S65T derivative of Green Fluorescent Protein.
7. The recombinant vector according to claim 5 wherein the recombinant DNA molecule has a DNA sequence encoding a Yeast Enhanced derivative of Green Fluorescent Protein.
8. The recombinant vector according to any preceding claim, wherein the vector is designed to autonomously replicate in a cell.

9. The recombinant vector according to any preceding claim, wherein the vector contains DNA from the 2 $\mu$  plasmid.
10. The recombinant vector according to any preceding claim, wherein the vector contains DNA encoding for part of the HO gene from chromosome IV of a yeast.
11. The recombinant vector according to claim 1, wherein the DNA vector is a PFA vector.
12. A cell containing a recombinant vector according to any one of claims 1 to 11.
13. A cell containing a recombinant vector according to claim 2 or 3.
14. The cell according to claims 12 or 13 wherein the cell is a yeast.
15. The yeast according to claim 14, wherein the yeast is *Saccharomyces cerevisiae*.
16. The yeast according to claim 15 which is FF18984 or Y486 in haploid form.
17. A method of detecting the presence of an agent that causes or potentiates DNA damage, the method comprising subjecting a cell according to claim 12 to an agent and monitoring the expression of the light emitting reporter protein from the cell, wherein an increase of the expression in the presence of the agent indicates that the agent causes or potentiates DNA damage.
18. The method according to claim 17, wherein the agent is further screened to assess whether it is safe to expose a living organism to the agent.
19. The method according to claim 17, wherein the agent is a candidate medicament, food additive or cosmetic.

20. The method according to claim 17, wherein the agent is a contaminant of water supplies.

21. The method according to claim 17, wherein the agent is a contaminant of industrial effluents.

22. The method according to claim 17, wherein expression of the light emitting reporter protein is measured from a whole cell.

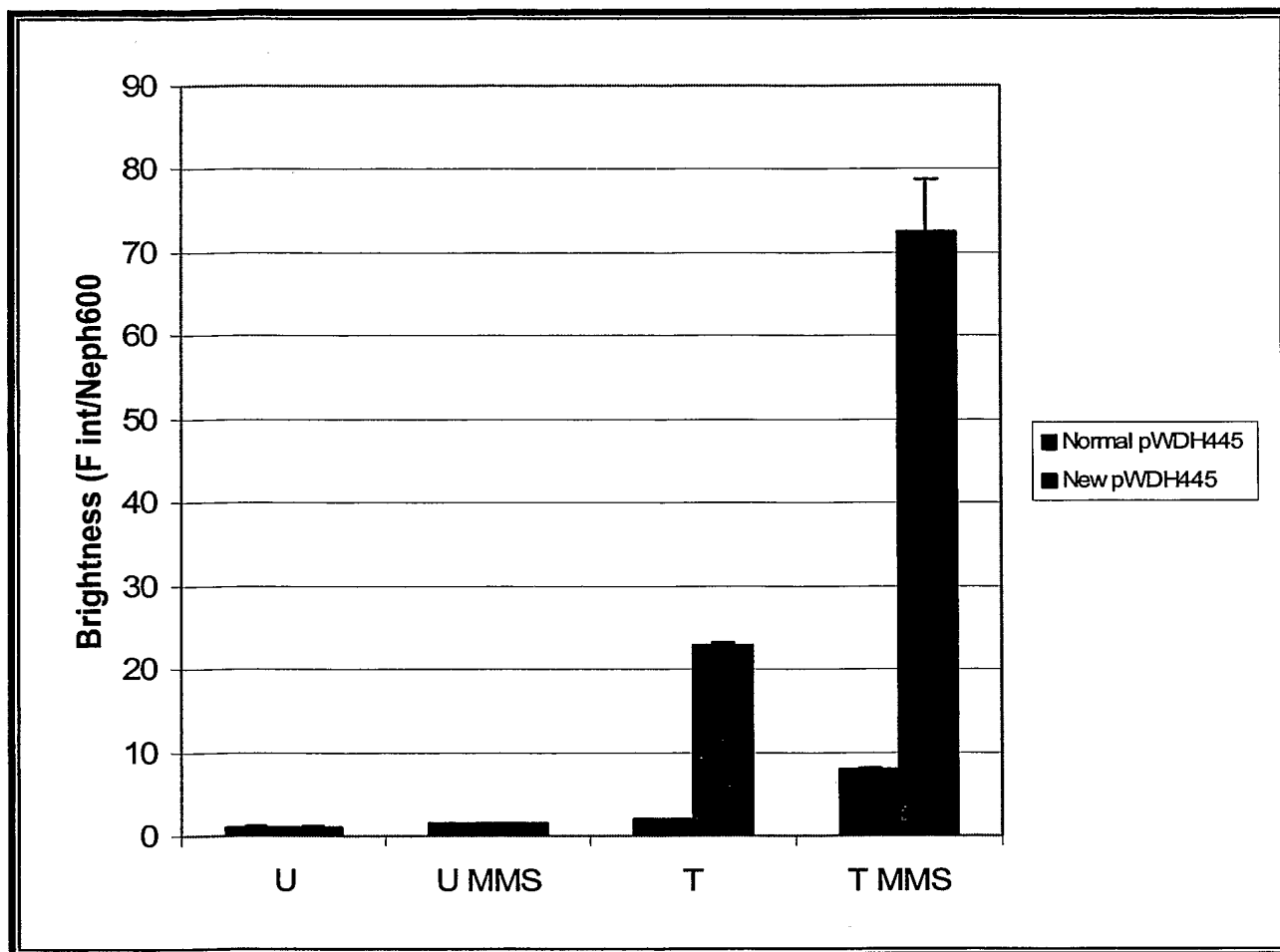
23. The method according to claim 22, wherein the light emitting reporter protein is Green Fluorescent Protein.

24. The method according to claim 23 comprising growing cells transformed with a recombinant vector according to claim 5, incubating the cells with the agent for a predetermined time and monitoring the expression of the Green Fluorescent Protein directly from a sample of the cells.

25. The method according to any one of claims 17 - 24 wherein the cells are grown in a low fluorescence growth medium.

26. The method according to claim 25 wherein the low fluorescence growth medium is F1 medium.

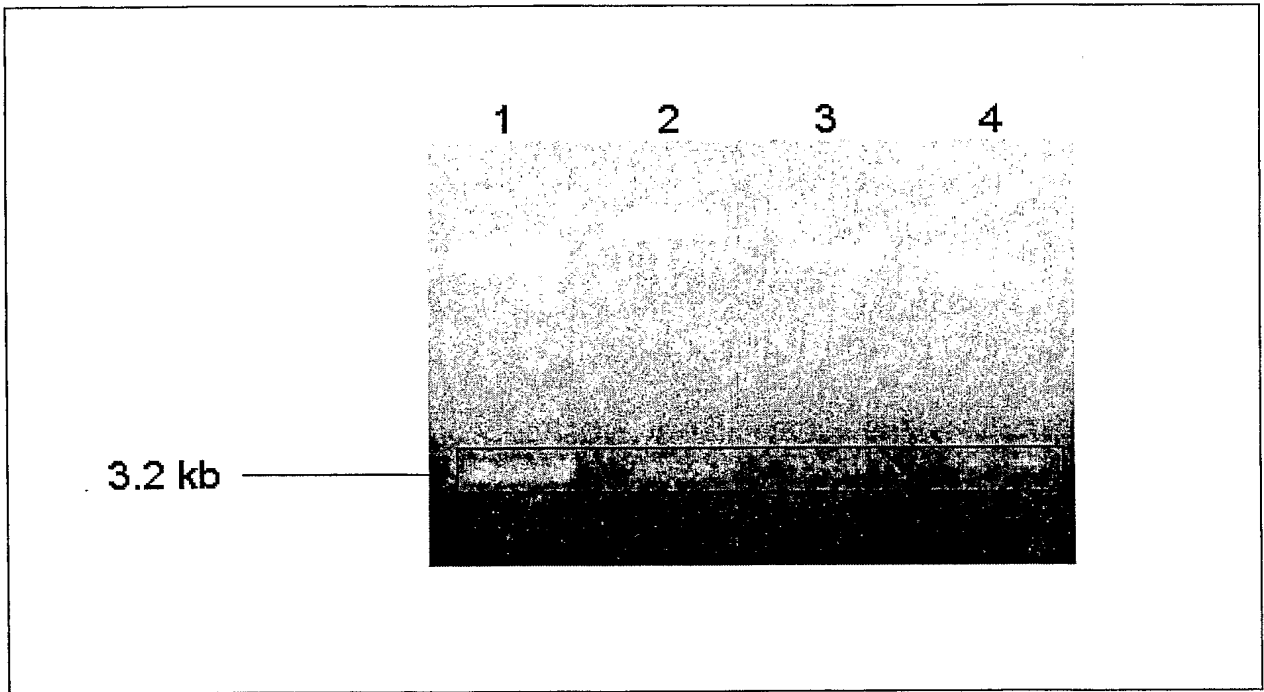


**FIG. 1**

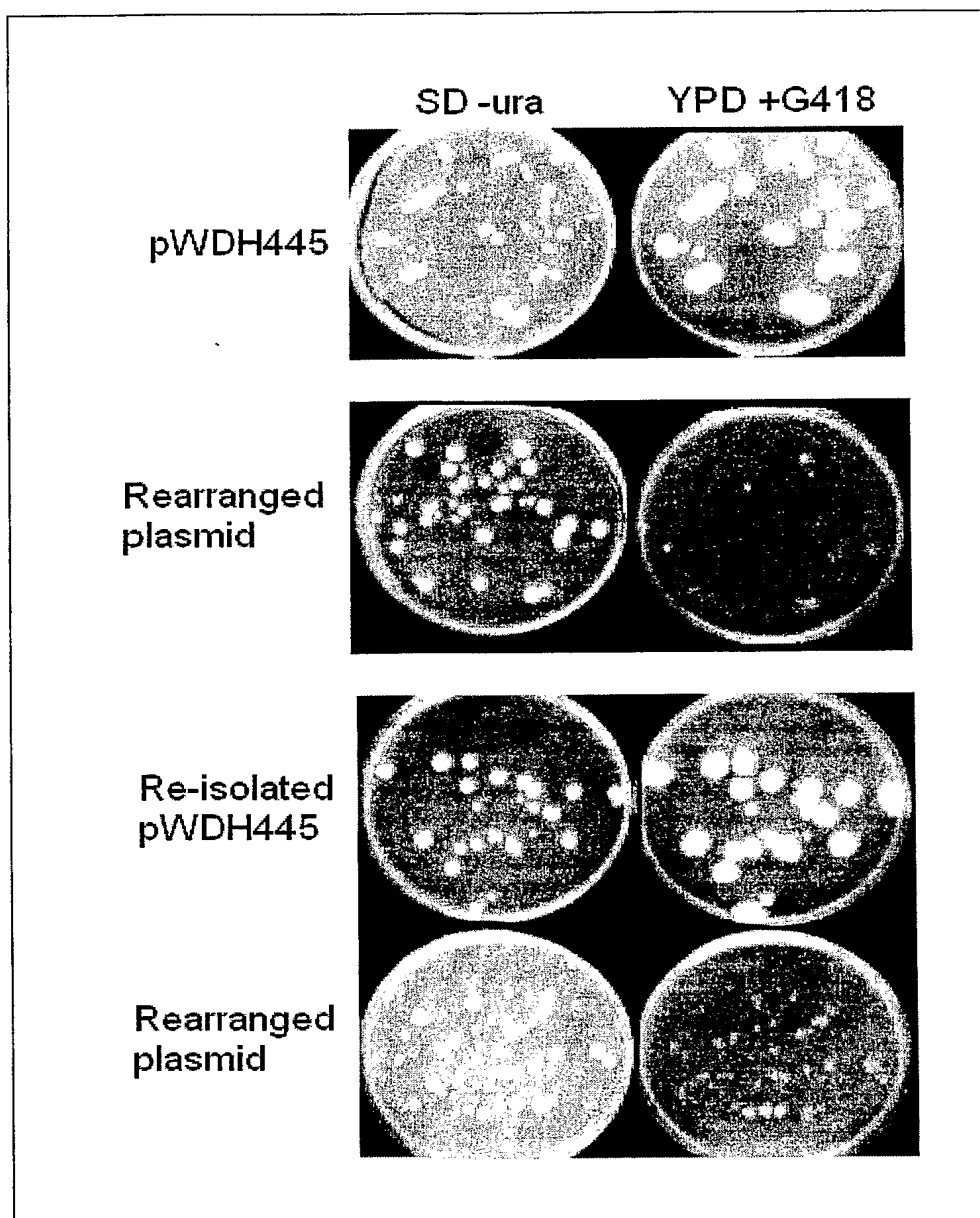




**FIG.2**



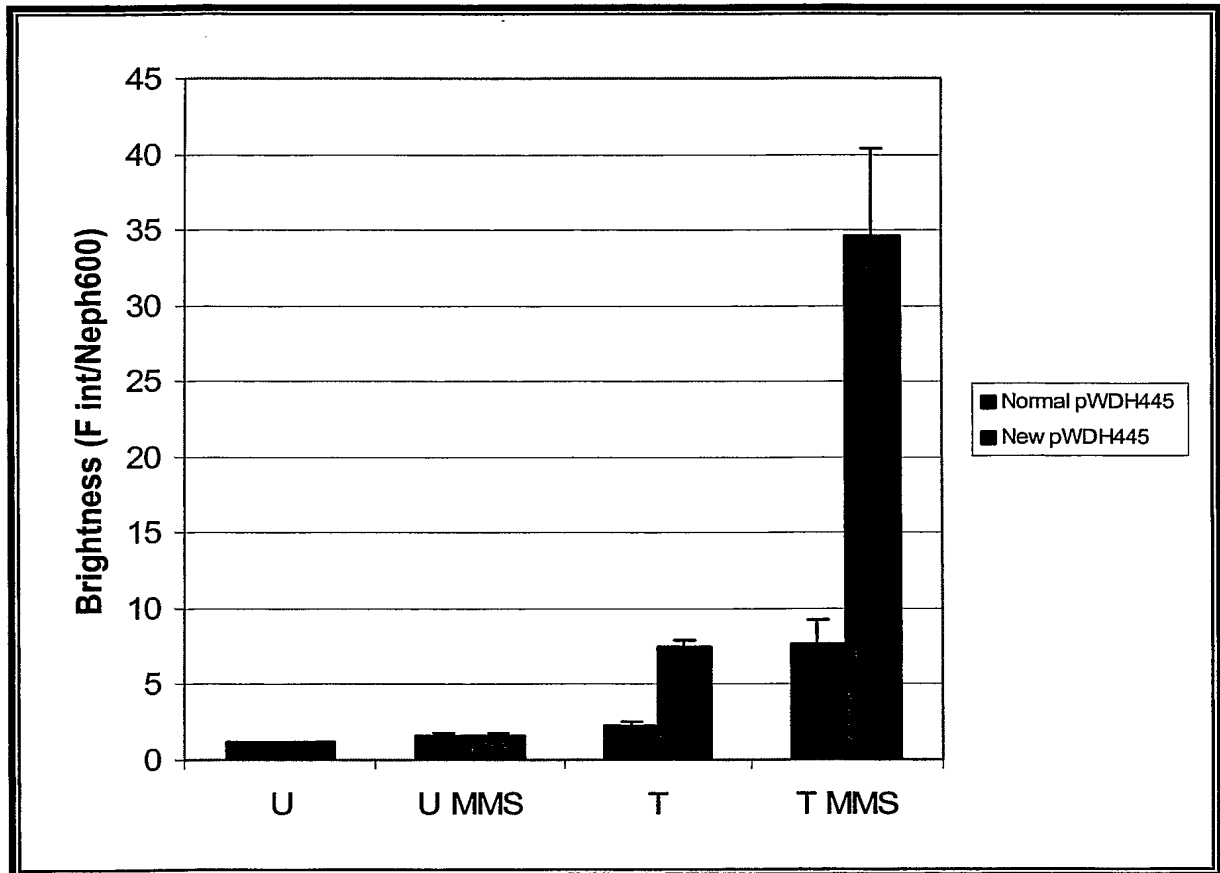


**FIG. 3**

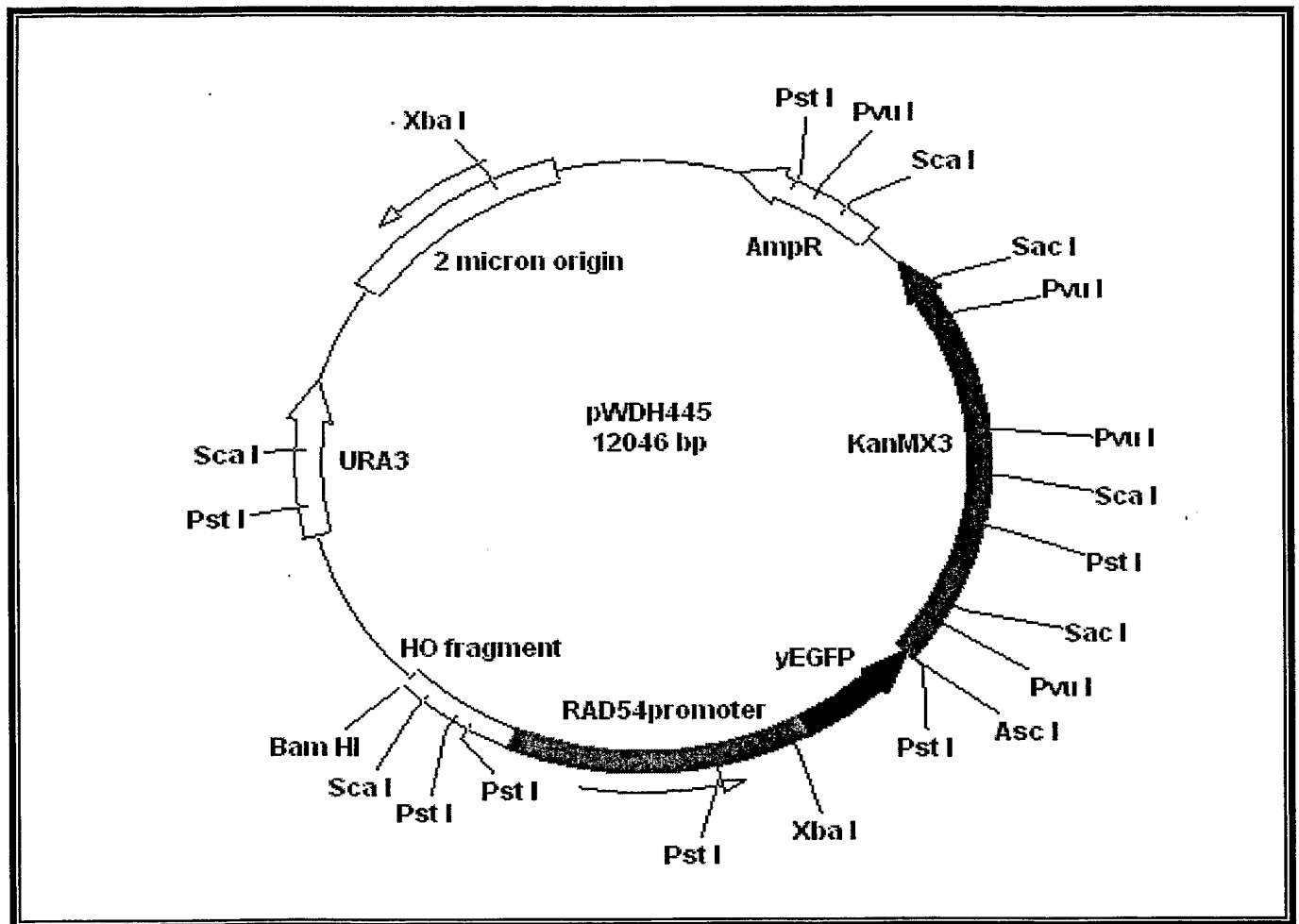


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**FIG. 4**

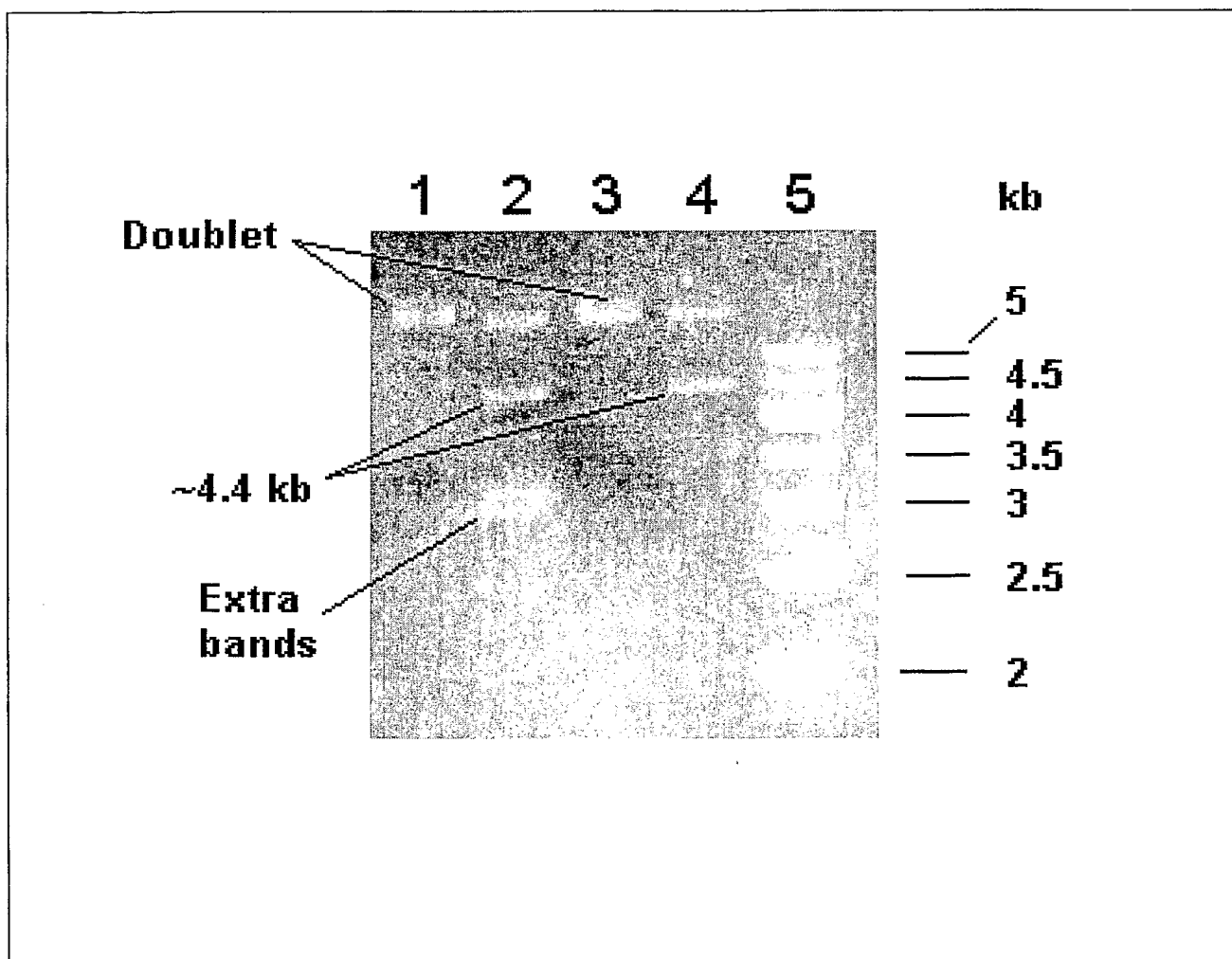




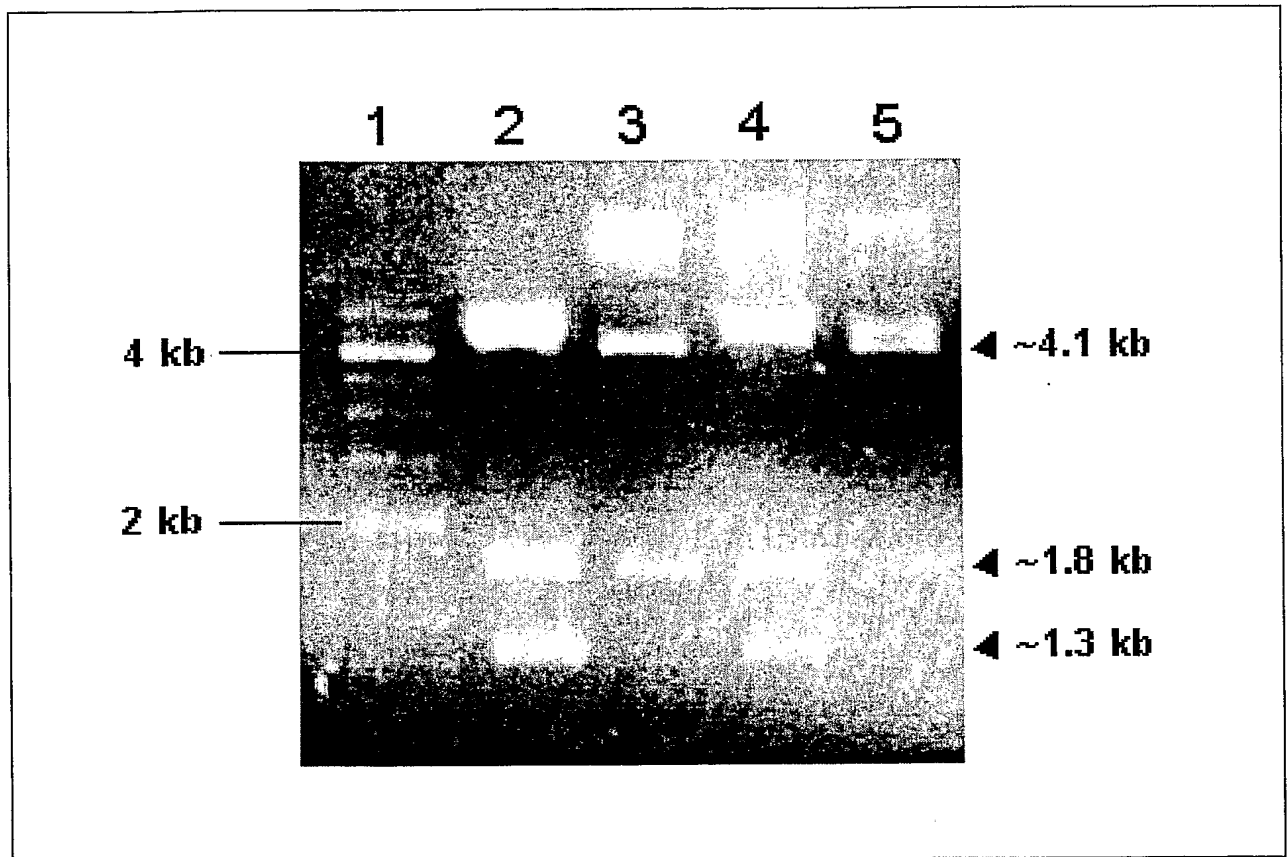
**FIG. 5**



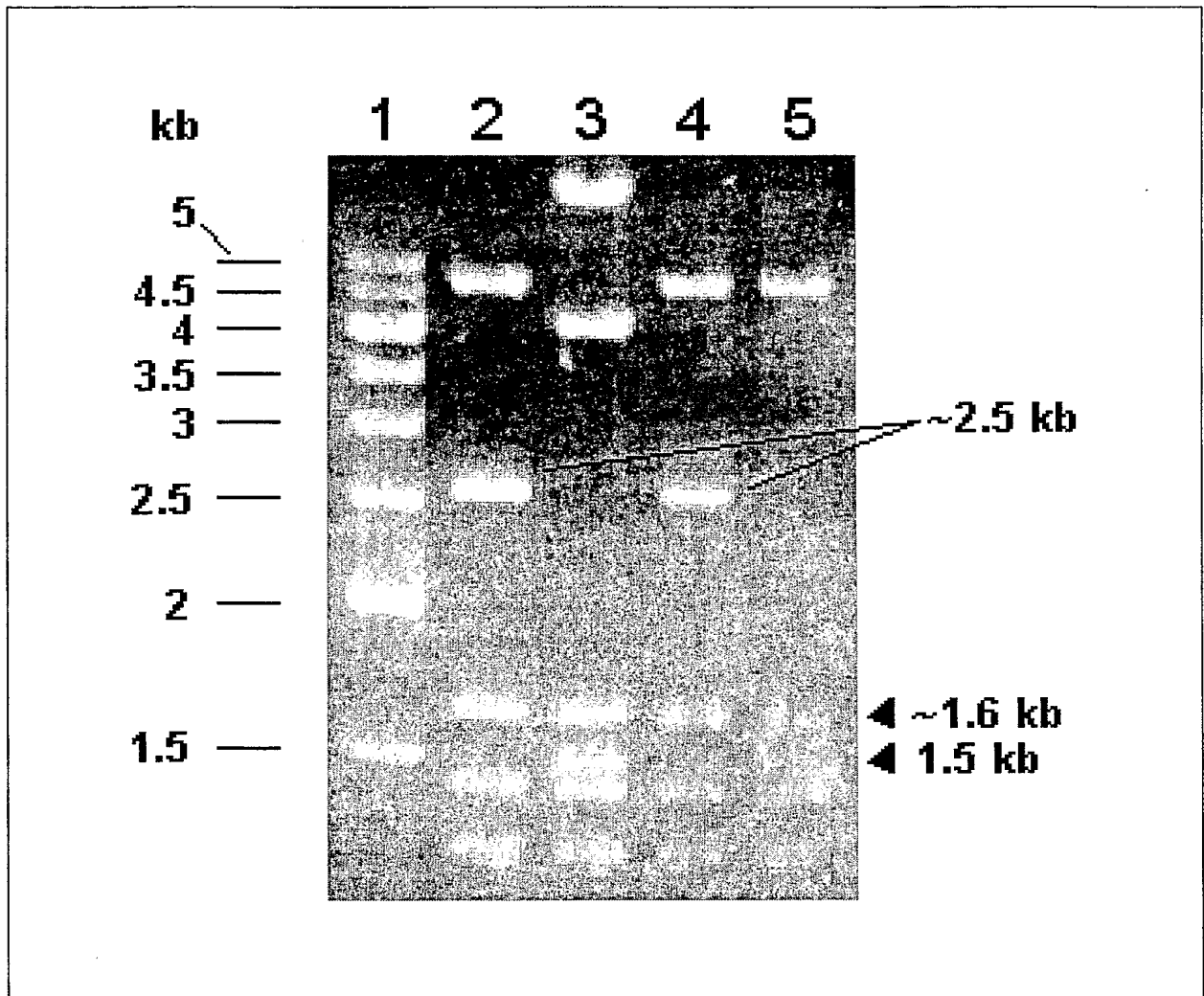


**FIG. 6**

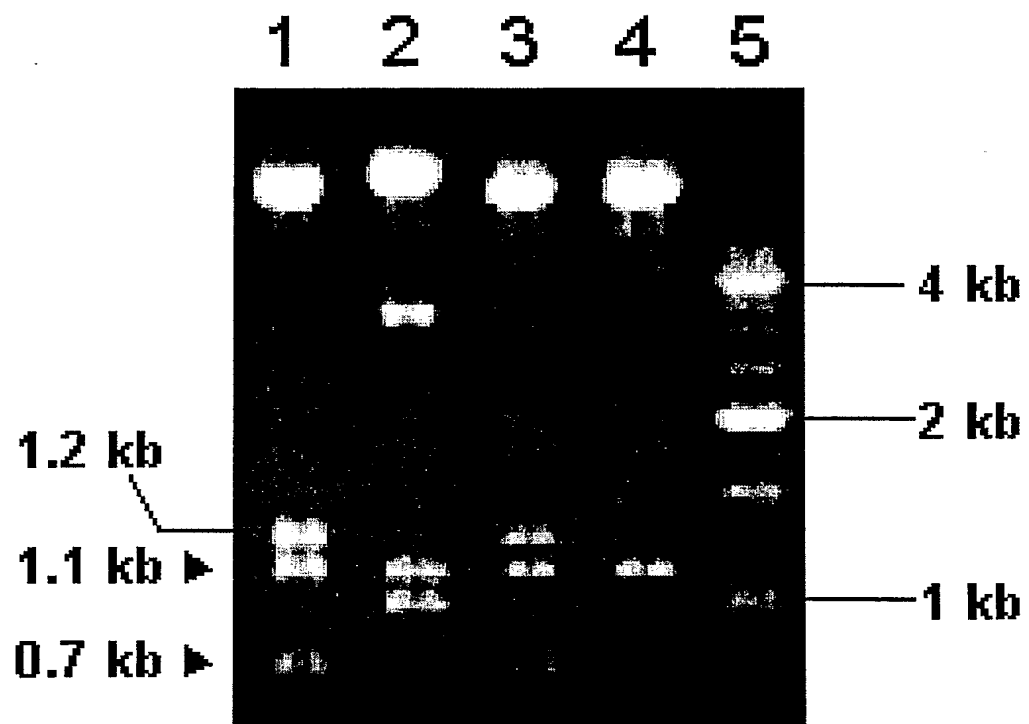


**FIG. 7**



**FIG. 8**



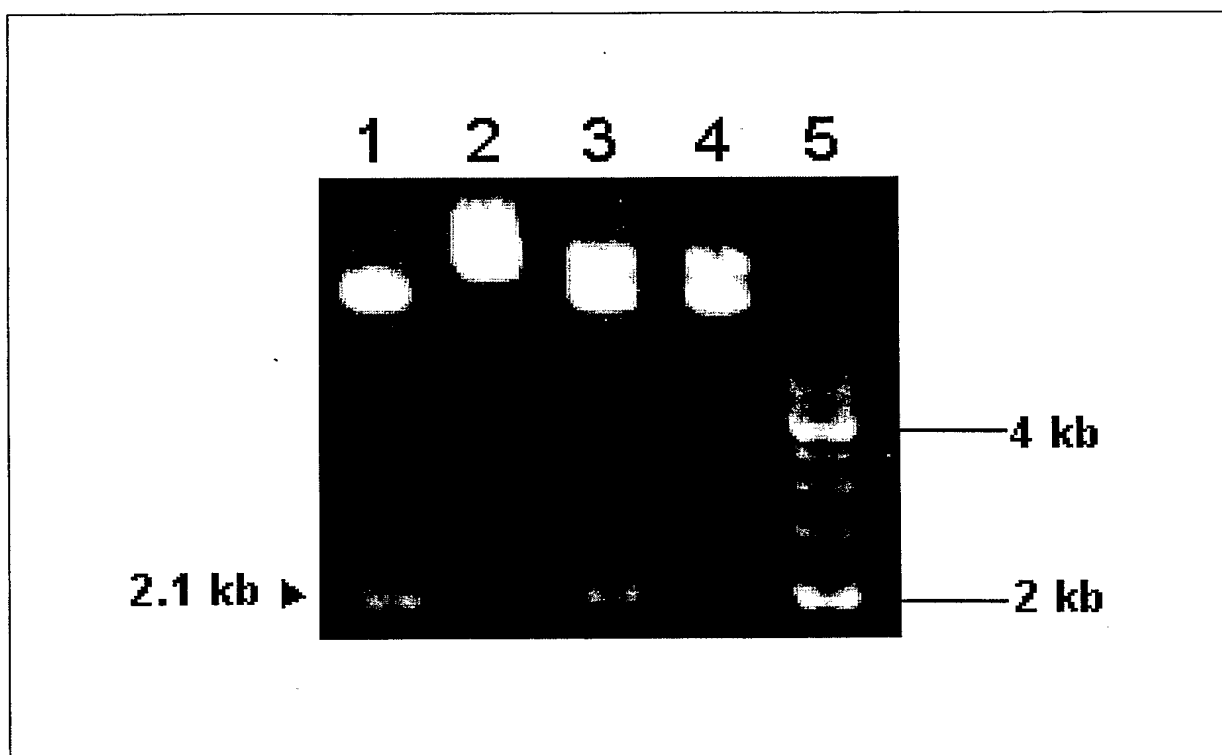
**FIG.9**





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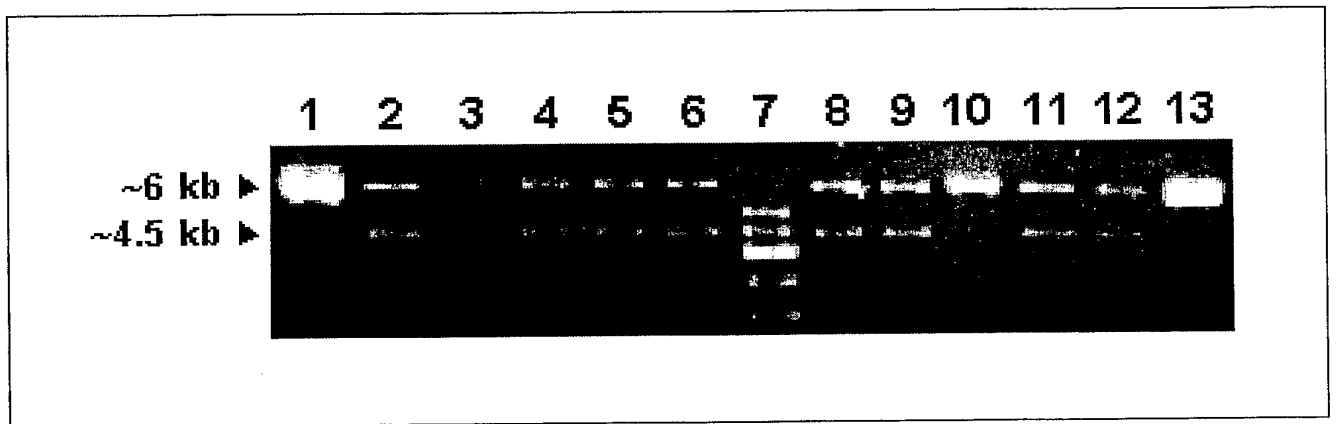
**FIG. 10**





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**FIG. 11**





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**FIG. 12**

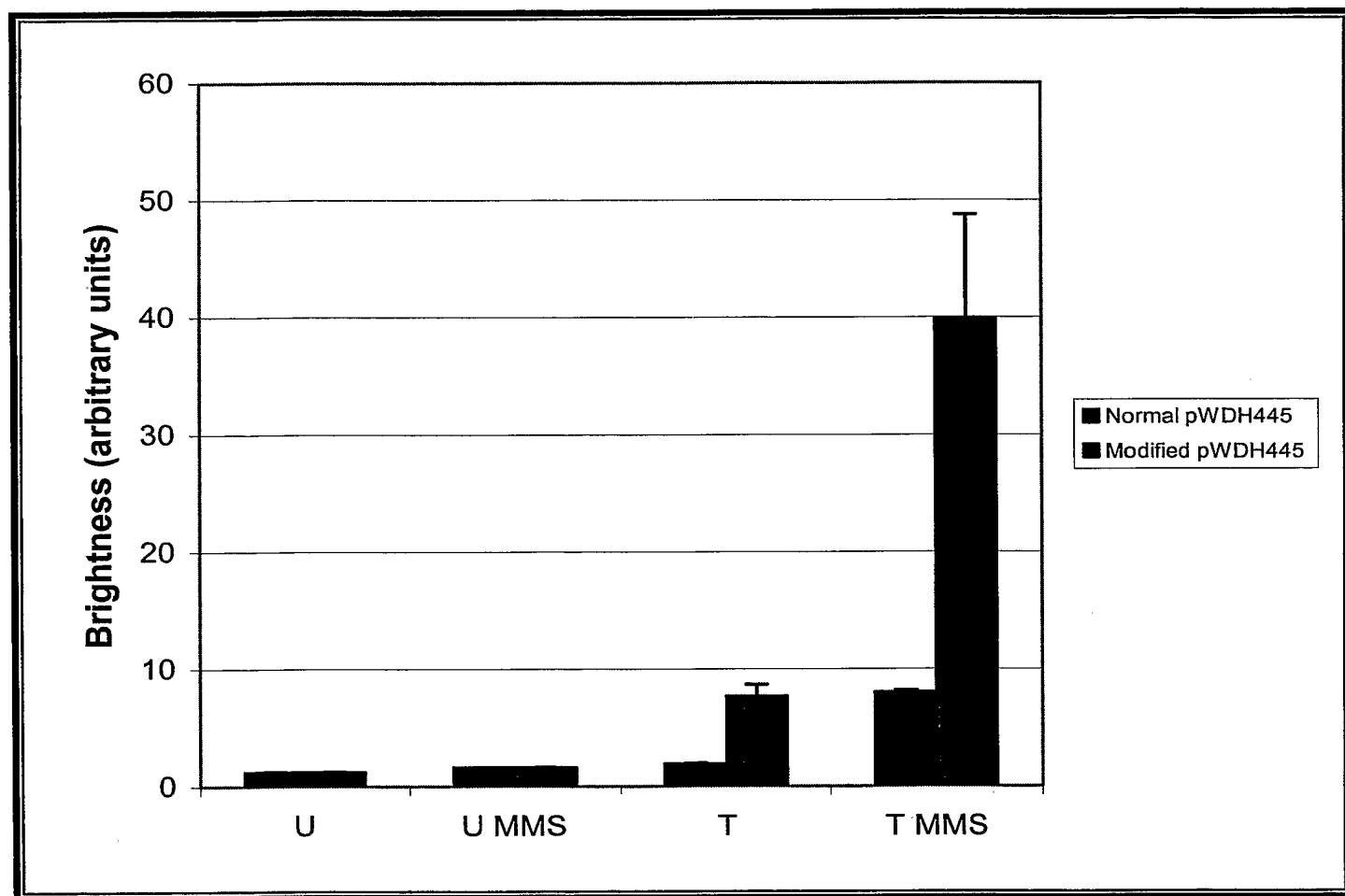
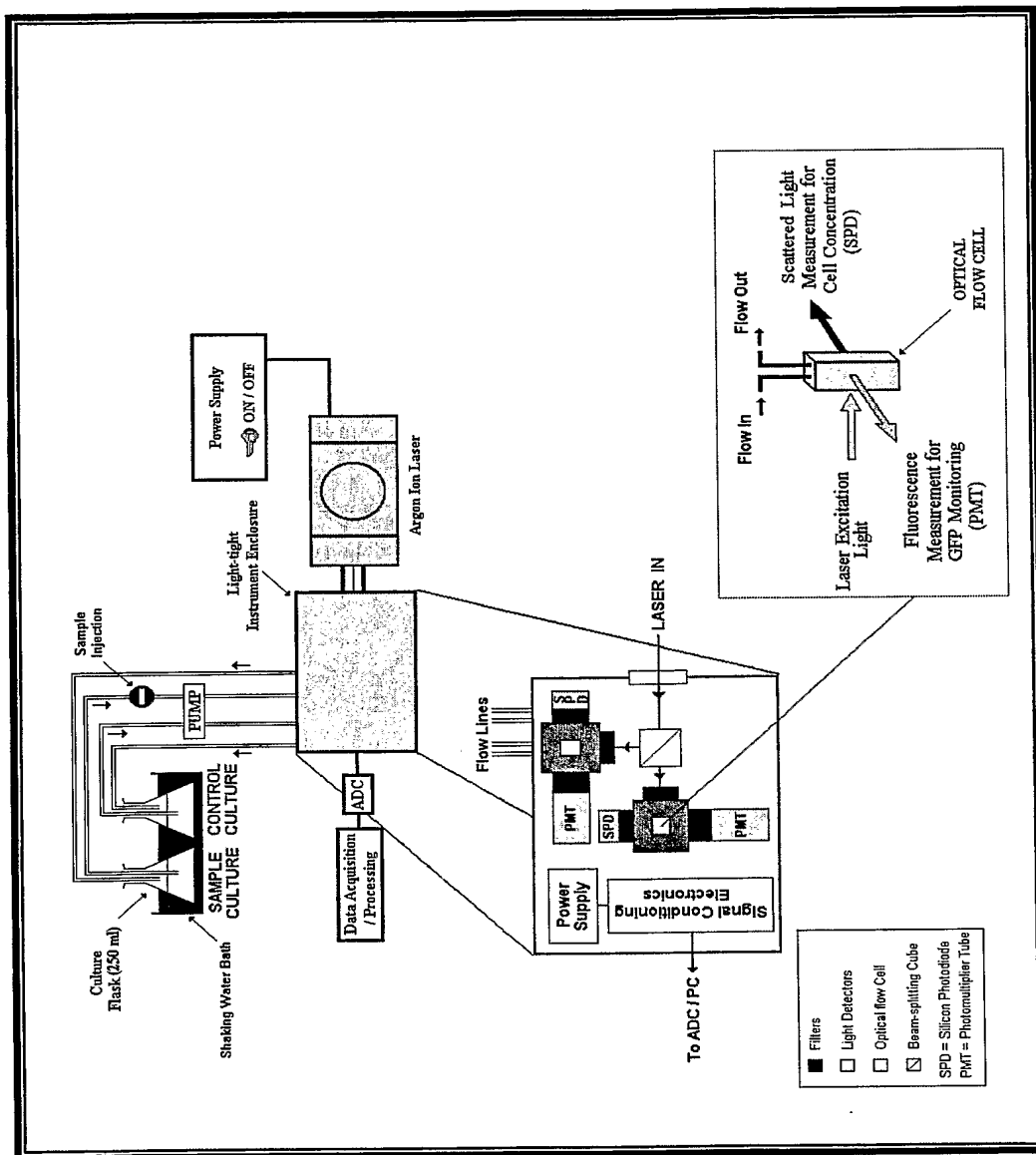


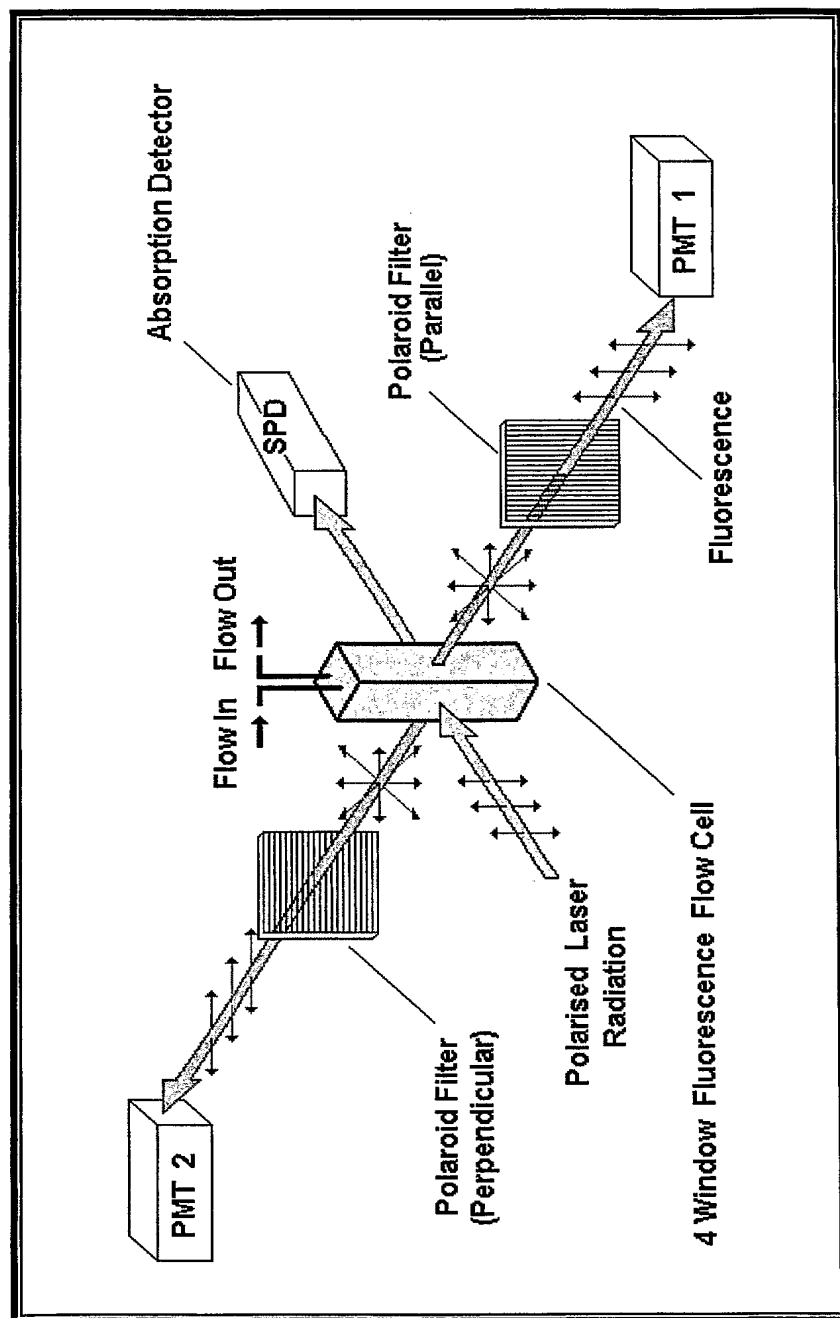


FIG. 13

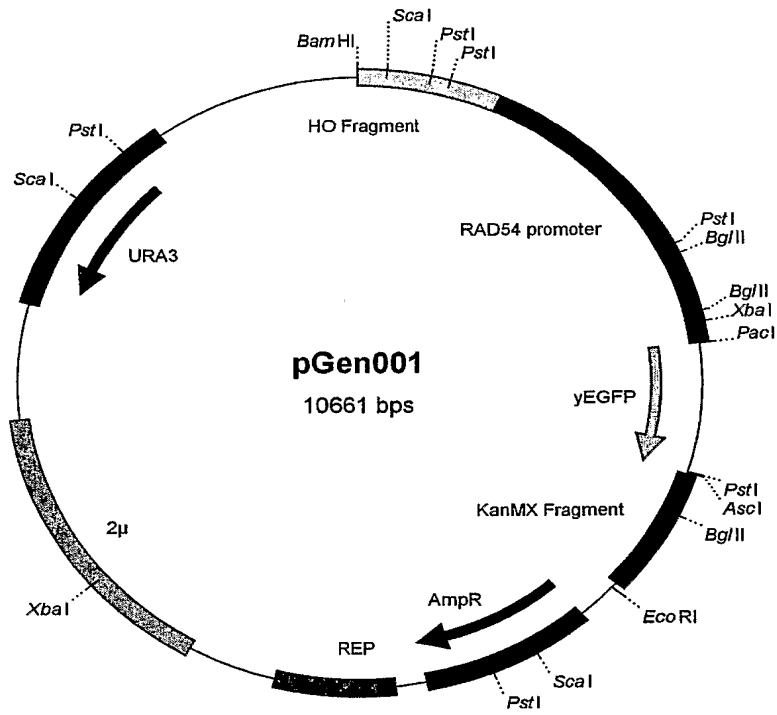






**FIG. 14**



**FIG. 15**







**Figure 16 continued**

[illegible]





**FIG. 17**

```

1   GATCCAAGCT ATCTACTGAG ATTTCTGGCT CTTTGTGTTGT ACTGTCACCT
51  AACCACAGAC CAAGCATCCA AGCCATACTT TTTACAGCAG GAGTTACAAG
101 GTCACTACGT CCAGTGAGAA ATTTAGATAA AACACCATTT CCTGCGAGTA
151 CTGGACCAAA TCTTATGCAG CTAGAAATTC TCAATTGAGC ATCAAGATAA
201 TCCAAATCTC TAACTTCAAT GTCAAAGTTG AAATATTCTC CTTTAGAGCG
251 CTCCATTTCT TCTATGAAGC GTTTTGCGGC AAATCACCT TCAACTGTCA
301 TTGGGAATGT CTTATGATGG TTTTTTGGAA TTATTATTAT CCTACCATCA
351 AGCGTCTGAC ATTGCTGCAG ATTTCTCCAT CTCACTTTAT ATTTGGTGGC
401 ATTTCTACCA CTTTTTTTCCA ACAGTGGTTT GGTAGGGACC CTGACTGACA
451 ATTTATGACC TGCAGTACAT TGTAATGCAA GACGCTGATA AACTGTTCTA
501 CGCCTGGGAT CTAACCTACC AGGTTACCT TCAAAAGCTC TGTGTTTGGT
551 TTTTTGCTGT ATATTATAGA TTTTCTGATA GCCCTGTGTG ACATTATATGA
601 CGCGGCGAGC GGAGCCATCT GCGCACATAA CGTAAGAGTT AGCCGTGACG
651 TTGCGATGT CTTTAATTTT ACCGTTAGCC ATCAGAATAG TCGTGTTTTC
701 AGAAAGCATT TTGATCCGAC ATACGATGAC CTCAATGATT TAGATTATGT
751 GTTGCACTTT TATAGACCTA CCAAAAATCC AGTGCGTACA CTAATACTTT
801 CATAAGATA CCTGAAACAA TAACCAGAAA GATCGGCAAA AAAATTTTTT
851 TTCTTTGCCG AGATCACAAA CCTACTATGA CGAAAAAGCT TGAAGTTTAG
901 ATGAGTAAGG AAAATACAAG TGACGCTTTT ATATGGTGCA AGGAACAAAA
951 ACTAAAAACA ACAAGGCAAA TGTGGATCTG TCATGTATGG CAACGACAGC
1001 AGGATGGCTC ACAAAAAAAG ACAAAAAAAG CTAAGGCAAA AGAACAAAGC
1051 TCCTCTCCTG CTCAAGAAAC GTATTGTTGA AAAACCACCG TCGTAAGAAA
1101 GTTTTTCTGT GACCTATAAT GGTTTAAAAT CGGCCCATTT TTTTCCCTC
1151 TTTTGTGGTC CAGTCTTTCT CATACTCGAG GGAAATTCGA CACAAACAGC
1201 GGAGAAGTGT GGCTAAACCG GCAAGTGCC TCAAGATCCA CAGAATAAC
1251 CGCACGAAC TGGCGTCCAG AAAGAGCCTG TTCCGAAAG AGAGAAACAG
1301 AGAAACGATC ATGATGGGAA AGCGGGGATT CGGCGAAGAA CGAGACTGGA
1351 AAGGGAAAAA GAGAAATACT GGTGGAAGTA TTCGGACCTT TGGCGAAGTC
1401 CGAACCTTGA AAACCCAAAG ATGATCGATG ATTCATTTTT CAATGCGCTA
1451 CGGTTCCCTG CGCTCGTGGG AACCCACGCG AAAACATATT ATTCGCTTCT
1501 CTCTGCTGAC AACTCCGGTT TACGTTATAC CGTATTAGGA TCACTATAAG
1551 GGTTCCTTCG GGAGGAGGGG GGAGGGGAAG AATGTACATC GTCATAAGGC
1601 CTTTATGGTG TGAAGTGGGT TTTGCGTGGA AAATTCGTTT TCAATGATAT
1651 AGAGCCACG CATATACGTA CATACTAGTG GCCAAAAGCG TGGGGTGGGC
1701 GGACAAAGCT AACTGGTAA AATACAGGAT TCTATGAACA ATAACAACAA
1751 CCAGCTCACG TTGCTGAACA GCCGAGGTCA GCCGATGCAA CCGAGGTTTC
1801 CAAAGTAGCA TTTCTGTGCT AGCTATGTCT GTAGGTTTAC ATTTAATGGT
1851 GCGTGGTTCC AGCTTCATGT GCTTGCATGT GATGTCCTGC AGATGGTAAG
1901 AAGATTCTGA AAGCCGCGCT AGGAGAAAAA TATTCTGCTC GAAGATCTGT
1951 CCTCTTAAGT AGAAAGCGTG AAATTGTTGC GTTCTTGCA TACTACTCAA
2001 CGCGTACGCA AATGCGTCTA CTGCACCTGC ATGATAAAGC TTATGTATCA
2051 AAAATTAAAC ATCTTGAAAA TACACAAGTG GTGCAAAGAT GTGTCACGTT
2101 CTGGACCTGA GTGGTGCCAT GTATGCTATT TAACATGCAA AGGGGAAGAC
2151 CCTTCCGCCT TACTGCAATA ATAAAAAGTA TTTTACGCGT TACCCAATAT
2201 AGCAAAGTTT CGCGCAAAAA AAAAAATAAA AAACAATTAC AAACAAAAAG
2251 AAAAAAAGG AAATAATAGA AGATCTAACT GAAGCGAAGG CCAAACTCT
2301 TCTCACTTGA CGTAATAGCC GATACAAAAT CTAGAGCAGC AACTTTCTC
2351 TTTCTTCACT AAAGCTGCTA CGAAAGTATA GAAAAATCAA ACGCTCAGAA
2401 CTTAGCTCTA TTTCAAGGTA CCATATATAT TTCCTTATAA CTGATGTTAA
2451 TTAACCTCTA AGGTGAAGAA TTATTCACTG GTGTTGTCCC AATTTTGGTT
2501 GAATTAGATG GTGATGTTAA TGGTCACAAA TTTTCTGTCT CCGGTGAAGG

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Fig 17 continued

2551	TGAAGGTGAT	GCTACTTACG	GTAAATTGAC	CTTAAAATTT	ATTTGTACTA
2601	CTGGTAAATT	GCCAGTTCCA	TGGCCAACCT	TAGTCACTAC	TTTCGGTTAT
2651	GGTGTTC AAT	GTTTTGCGAG	ATACCCAGAT	CATATGAAAC	AACATGACTT
2701	TTTCAAGTCT	GCCATGCCAG	AAGGTTATGT	TCAAGAAAGA	ACTATTTTTT
2751	TCAAAGATGA	CGGTA ACTAC	AAGACCAGAG	CTGAAGTCAA	GTTTGAAGGT
2801	GATACCTTAG	TTAATAGAAT	CGAATTAAAA	GGTATTGATT	TTAAAGAAGA
2851	TGGTAACATT	TTAGGTCACA	AAATTGGAATA	CAACTATAAC	TCTCACAATG
2901	TTTACATCAT	GGCTGACAAA	CAAAAGAATG	GTATCAAAGT	TAACTTCAAA
2951	ATTAGACACA	ACATTGAAGA	TGGTTC TGTT	CAATTAGCTG	ACCATTATCA
3001	ACAAAATACT	CCAATTGGTG	ATGGTCCAGT	CTTGTTACCA	GACAACCATT
3051	ACTTATCCAC	TCAATCTGCC	TTATCCAAAG	ATCCAAACGA	AAAGAGAGAC
3101	CACATGGTCT	TGTTAGAATT	TGTTACTGCT	GCTGGTATTA	CCCATGGTAT
3151	GGATGAATTG	TACAAATAAC	TGCAGGGCGC	GCCACTTCTA	AATAAGCGAA
3201	TTTCTTATGA	TTTATGATTT	TTATTATTAA	ATAAGTTATA	AAAAAAATAA
3251	GTGTATACAA	ATTTTAAAGT	GACTCTTAGG	TTTTAAACG	AAAATTCTTA
3301	TTCTTGAGTA	ACTCTTTCCT	GTAGGTCAGG	TTGCTTTCTC	AGGTATAGTA
3351	TGAGGTCGCT	CTTATTGACC	ACACCTCTAC	CGGCAGATCC	GCTATGGATA
3401	ACAGGGTAAT	ATAGATCTGC	CCGCCGGGAA	GGCGAACCCG	ATCGGATGCA
3451	TCCTCTCTGC	TGCCATGATG	CTGAAGTTGT	CGTTGAACAT	GGTTGCTGCC
3501	GGCGAGGCGG	TCGAGCAGGC	AGTGCAGGAG	GTGTTGGACT	CGGGAGTCAG
3551	AACGGGCGAC	CTGCTCGGCT	CGAGCTCCAC	TTCGGAGGTT	GGCGACGCCA
3601	TTGCGCTTGC	AGTTAAGGAA	GCCTTGCGCA	GGCAATCCGC	AGCTGGTCTG
3651	AGCTAGCCTC	GAGGACCCTT	CTCTTTAGAC	TATTCTACTC	TTATGCACGT
3701	AAAAAATCT	AGGAAATATG	TATTA ACTAG	GAGTAAAATA	ACCGGCTAGT
3751	GGCATTCATA	TAGCCGTCTG	TTTACATCTA	CATCACACAT	TTTCGAGTGA
3801	TATCTCGCAA	CGTTGGCGTT	AAATAGGCAG	TCAATGGCCC	GACCATTTCTA
3851	TGGTGTTTAG	GTCGATGCCA	TCTTTGTACG	TTTAGCTTAT	CGATGATAAG
3901	CTGTCAAACA	TGAGAATTCT	TGAAGACGAA	AGGGCCTCGT	GATACGCCTA
3951	TTTTTATAGG	TTAATGTCAT	GATAATAATG	GTTTTCTTAGA	CGTCAGGTGG
4001	CACTTTTCCG	GGAAATGTGC	GCGGAACCCC	TATTTGTTTA	TTTTTCTAAA
4051	TACATTCAAA	TATGTATCCG	CTCATGAGAC	AATAACCCCTG	ATAAATGCTT
4101	CAATAATATT	GAAAAAGGAA	GAGTATGAGT	ATTCAACATT	TCCGTGTCGC
4151	CCTTATTTCC	TTTTTTGCGG	CATTTTGCCT	TCCTGTTTTT	GCTCACCCAG
4201	AAACGCTGGT	GAAAGTAAAA	GATGCTGAAG	ATCAGTTGGG	TGCACGAGTG
4251	GGTTACATCG	AACTGGATCT	CAACAGCGGT	AAGATCCTTG	AGAGTTTTTCG
4301	CCCCGAAGAA	CGTTTTTCAA	TGATGAGCAC	TTTTAAAGTT	CTGCTATGTG
4351	GCGCGGTATT	ATCCCGTGTT	GACGCCGGGC	AAGAGCAACT	CGGTCGCCGC
4401	ATACACTATT	CTCAGAATGA	CTTGTTGAG	TACTCACCAG	TCACAGAAAA
4451	GCATCTTACG	GATGGCATGA	CAGTAAGAGA	ATTATGCAGT	GCTGCCATAA
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4551	CCGAAGGAGC	TAACCGCTTT	TTTGCACAAC	ATGGGGGATC	ATGTA ACTCG
4601	CCTTGATCGT	TGGGAACCGG	AGCTGAATGA	AGCCATACCA	AACGACGAGC
4651	GTGACACCAC	GATGCCTGCA	GCAATGGCAA	CAACGTTGCG	CAA ACTATTA
4701	ACTGGCGAAC	TACTTACTCT	AGCTTCCCGG	CAACAATTAA	TAGACTGGAT
4751	GGAGGCGGAT	AAAGTTGCAG	GACCACTTCT	GCGCTCGGCC	CTTCCGGCTG
4801	GCTGGTTTAT	TGCTGATAAA	TCTGGAGCCG	GTGAGCGTGG	GTCTCGCGGT
4851	ATCATTTGCAG	CACTGGGGCC	AGATGGTAAG	CCCTCCCGTA	TCGTAGTTAT
4901	CTACACGACG	GGGAGTCAGG	CAACTATGGA	TGAACGAAAT	AGACAGATCG
4951	CTGAGATAGG	TGCCTCACTG	ATTAAGCATT	GGTAACTGTC	AGACCAAGTT
5001	TACTCATATA	TACTTTTAGAT	TGATTTAAAA	CTTCATTTTT	AATTTAAAAG
5051	GATCTAGGTG	AAGATCCTTT	TTGATAATCT	CATGACCAAA	ATCCCTTAAC
5101	GTGAGTTTTT	GTTCCACTGA	CGGTCAGACC	CCGTAGAAAA	GATCAAAGGA
5151	TCTTCTTGAG	ATCCTTTTTT	TCTGCGCGTA	ATCTGCTGCT	TGCAACAAA
5201	AAAACCACCG	CTACCAGCGG	TGGTTTGTTT	GCCGGATCAA	GAGCTACCAA



Fig 17 continued

5251	CTCTTTTTCC	GAAGGTAAC	GGCTTCAGCA	GAGCGCAGAT	ACCAAATACT
5301	GTCCTTCTAG	TGTAGCCGTA	GTTAGGCCAC	CACTTCAAGA	ACTCTGTAGC
5351	ACCGCCTACA	TACCTCGCTC	TGCTAATCCT	GTTACCAAGT	GCTGCTGCCA
5401	GTGGCGATAA	GTCGTGTCTT	ACCGGGTTGG	ACTCAAGACG	ATAGTTACCG
5451	GATAAGGCGC	AGCGGTGCGG	CTGAACGGGG	GGTTCGTGCA	CACAGCCCAG
5501	CTTGAGCGA	ACGACCTACA	CCGAACGAG	ATACCTACAG	CGTGAGCTAT
5551	GAGAAAGCGC	CACGCTTCCC	GAAGGGAGAA	AGGCGGACAG	GTATCCGGTA
5601	AGCGGCAGGG	TCGGAACAGG	AGAGCGCACG	AGGGAGCTTC	CAGGGGGAAA
5651	CGCCTGGTAT	CTTTATAGTC	CTGTGCGGTT	TCGCCACCTC	TGACTTGAGC
5701	GTGATTTTTT	GTGATGCTCG	TCAGGGGGGC	GGAGCCTATG	GAAAAACGCC
5751	AGCAACGCGG	CCTTTTTTACG	GTTTCTGGCC	TTTTGCTGGC	CTTTTGCTCA
5801	CATGTTCTTT	CCTGCGTTAT	CCCCTGATT	TGTGGATAAC	CGTATTACCG
5851	CCTTTGAGTG	AGCTGATACC	GCTCGCCGCA	GCCGAACGAC	CGAGCGCAGC
5901	GAGTCAGTGA	GCGAGGAAGC	GGAAGAGCGC	CTGATGCGGT	ATTTTCTCCT
5951	TACGCATCTG	TGCGGTATTT	CACACCGCAT	ATGGTGCACT	CTCAGTACAA
6001	TCTGCTCTGA	TGCCGCATAG	TTAAGCCAGT	ATACACTCCG	CTATCGCTAC
6051	GTGACTGGGT	CATGGCTGCG	CCCCGACACC	CGCCAACACC	CGCTGACGCG
6101	CCCTGACGGG	CTTGTCTGCT	CCCGGCATCC	GCTTACAGAC	AAGCTGTGAC
6151	CGTCTCCGGG	AGCTGCATGT	GTCAGAGGTT	TTCACCGTCA	TCACCGAAAC
6201	GCGCGAGGCA	GAGCTTTGAA	GAAAAATGCG	CCTTATTCAA	TCTTTGCTAT
6251	AAAAAATGGC	CCAAAATCTC	ACATTGGAAG	ACATTTGATG	ACCTCATTTC
6301	TTTCAATGAA	GGGCCTAACG	GAGTTGACTA	ATGTTGTGGG	AAATTGGAGC
6351	GATAAGCGTG	CTTCTGCCGT	GGCCAGGACA	ACGTATACTC	ATCAGATAAC
6401	AGCAATACCT	GATCACTACT	TCGCACTAGT	TTCTCGGTAC	TATGCATATG
6451	ATCCAATATC	AAAGGAAATG	ATAGCATTGA	AGGATGAGAC	TAATCCAATT
6501	GAGGAGTGGC	AGCATATAGA	ACAGCTAAAG	GGTAGTGCTG	AAGGAAGCAT
6551	ACGATACCCC	GCATGGAATG	GGATAATATC	ACAGGAGGTA	CTAGACTACC
6601	TTTCATCCTA	CATAAATAGA	CGCATATAAG	TACGCATTTA	AGCATAAACA
6651	CGCACTATGC	CGTTCTTCTC	ATGTATATAT	ATATACAGGC	AACACGCAGA
6701	TATAGGTGCG	ACGTGAACAG	TGAGCTGTAT	GTGCGCAGCT	CGCGTTGCAT
6751	TTTCGGAAGC	GCTCGTTTTT	GGAAACGCTT	TGAAGTTCCT	ATTCGGAAGT
6801	TCCTATTCTC	TAGAAAGTAT	AGGAACTTCA	GAGCGCTTTT	GAAAACCAAA
6851	AGCGCTCTGA	AGACGCACTT	TCAAAAAACC	AAAAACGCAC	CGGACTGTAA
6901	CGAGCTACTA	AAATATTGCG	AATACCGCTT	CCACAAACAT	TGCTCAAAAG
6951	TATCTCTTTG	CTATATATCT	CTGTGCTATA	TCCCTATATA	ACCTACCCAT
7001	CCACCTTTTC	CTCCTTGAAC	TTGCATCTAA	ACTCGACCTC	TACATTTTTT
7051	ATGTTTATCT	CTAGTATTAC	TCTTTAGACA	AAAAAATTGT	AGTAAGAACT
7101	ATTCATAGAG	TGAATCGAAA	ACAATACGAA	AATGTAAACA	TTTCCTATAC
7151	GTAGTATATA	GAGACAAAAT	AGAAGAAACC	GTTTATAATT	TTCTGACCAA
7201	TGAAGAATCA	TCAACGCTAT	CACTTTCTGT	TCACAAAGTA	TGCGCAATCC
7251	ACATCGGTAT	AGAATATAAT	CGGGGATGCC	TTTATCTTGA	AAAAATGCAC
7301	CCGCAGCTTC	GCTAGTAATC	AGTAAACGCG	GGAAGTGGAG	TCAGGCTTTT
7351	TTTATGGAAG	AGAAAAATAGA	CACCAAAGTA	GCCTTCTTCT	AACCTTAACG
7401	GACCTACAGT	GCAAAAAGTT	ATCAAGAGAC	TGCATTATAG	AGCGCACAAA
7451	GGAGAAAAAA	AGTAATCTAA	GATGCTTTGT	TAGAAAAATA	GCGCTCTCGG
7501	GATGCATTTT	TGTAGAACAA	AAAAGAAGTA	TAGATTCTTT	GTTGGTAAAA
7551	TAGCGCTCTC	GCGTTGCATT	TCTGTTCTGT	AAAAATGCAG	CTCAGATTCT
7601	TTGTTTGAAA	AATTAGCGCT	CTCGCGTTGC	ATTTTTGTTT	TACAAAAATG
7651	AAGCACAGAT	TCTTCGTTGG	TAAAATAGCG	CTTTCGCGTT	GCATTTCTGT
7701	TCTGTAAAAA	TGCAGCTCAG	ATTCTTTGTT	TGAAAAATTA	GCGCTCTCGC
7751	GTTGCATTTT	TGTTCTACAA	AATGAAGCAC	AGATGCTTCG	TTCTGCGGTA
7801	AAGCTCATCA	GCGTGGTCGT	GAGCGATTTC	ACAGATGTCT	GCCTGTTCAT
7851	CCGCGTCCAG	CTCGTTGAGT	TTCTCCAGAA	GCGTTAATGT	CTGGCTTCTG
7901	ATAAAGCGGG	CCATGTTAAG	GGCGGTTTTT	TCCTGTTTGG	TCACTGATGC



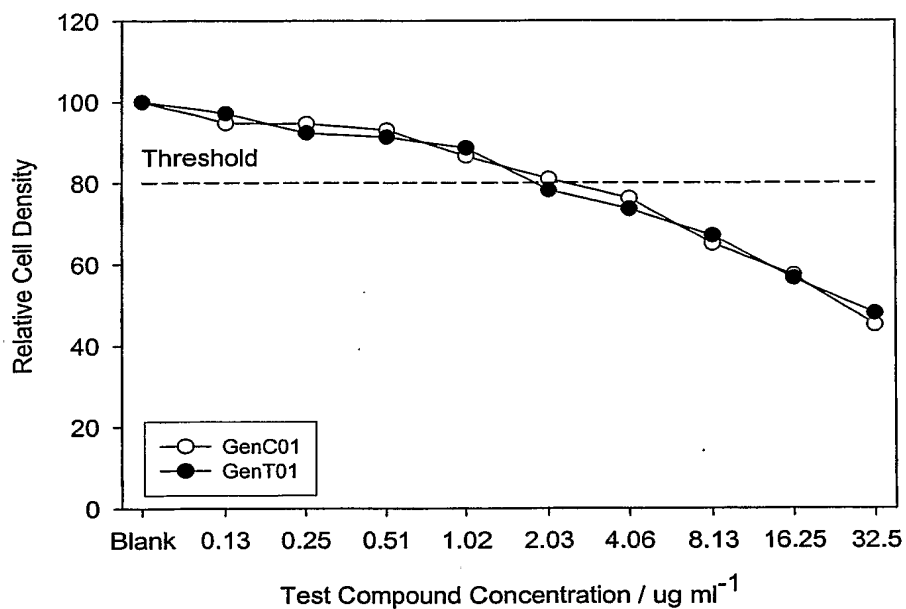
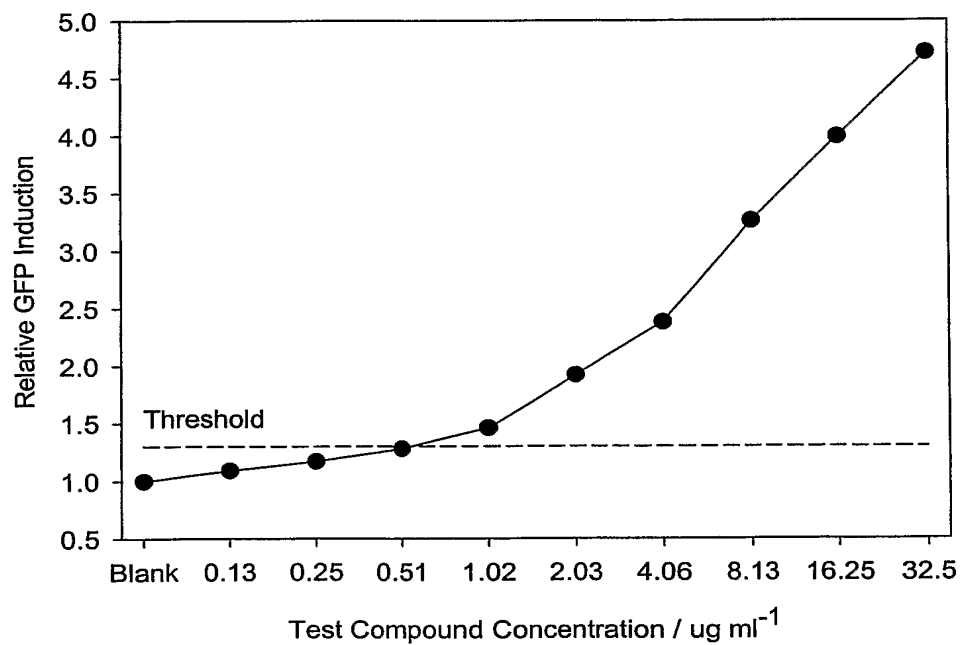
Figure 17 continued

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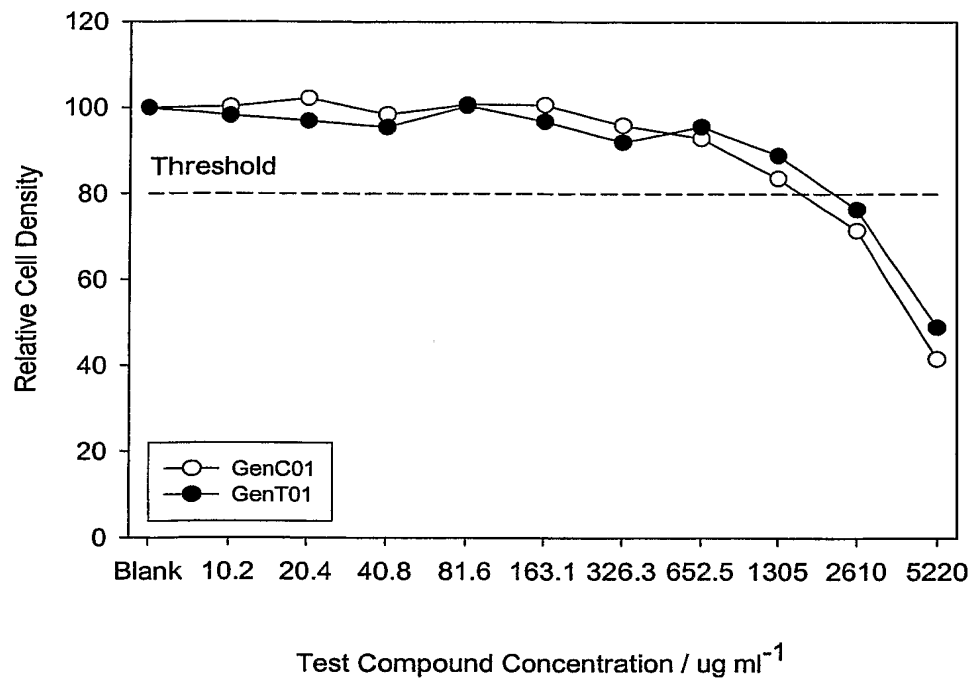
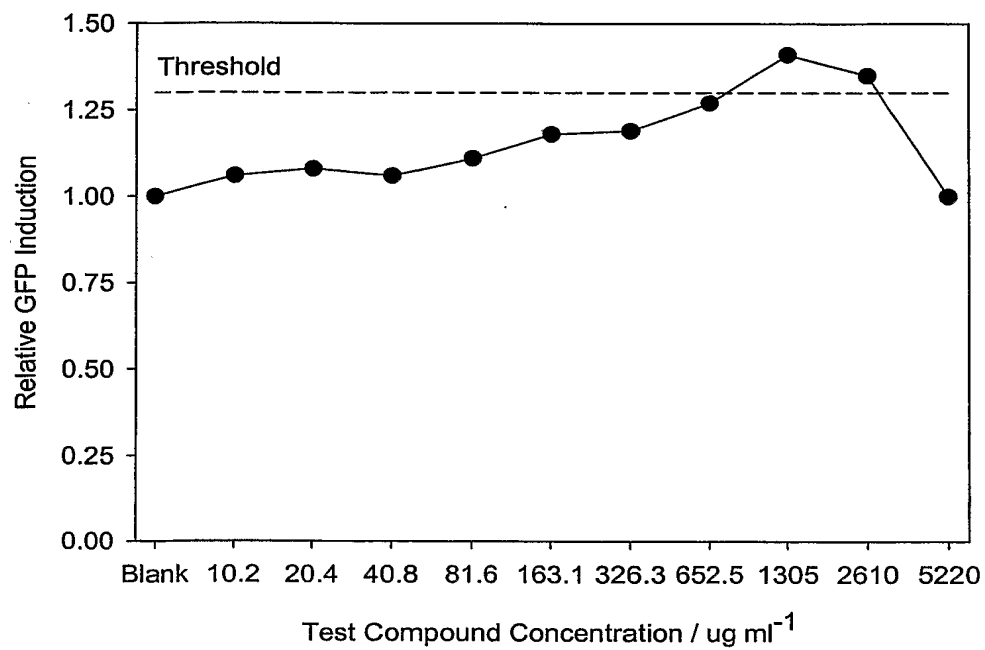
7951	CTCCGTGTAA	GGGGGATTTT	TGTTTCATGGG	GGTAATGATA	CCGATGAAAC
8001	GAGAGAGGAT	GCTCACGATA	CGGGTTACTG	ATGATGAACA	TGCCCGGTTA
8051	CTGGAACGTT	GTGAGGGTAA	ACAACCTGGCG	GTATGGATGC	GGCGGGACCA
8101	GAGAAAAATC	ACTCAGGGTC	AATGCCAGCG	CTTCGTTAAT	ACAGATGTAG
8151	GTGTTCCACA	GGGTAGCCAG	CAGCATCCTG	CGATGCAGAT	CCGGAACATA
8201	ATGGTGCAGG	GCGCTGACTT	CCGCGTTTCC	AGACTTTACG	AAACACGGAA
8251	ACCGAAGACC	ATTCATGTTG	TTGCTCAGGT	CGCAGACGTT	TTGCAGCAGC
8301	AGTCGCTTCA	CGTTCGCTCG	CGTATCGGTG	ATTCATTCTG	CTAACCAGTA
8351	AGGCAACCCC	GCCAGCCTAG	CCGGGTCTCT	AACGACAGGA	GCACGATCAT
8401	GCGCACCCGT	GGCCAGGACC	CAACGCTGCG	GGGGGGGGGG	GGGTTTTCTT
8451	TCCAATTTTT	TTTTTTTTCGT	CATTATAGAA	ATCATTACGA	CCGAGATTCC
8501	CGGGTAATAA	CTGATATAAT	TAAATTGAAG	CTCTAATTTG	TGAGTTTAGT
8551	ATACATGCAT	TTACTTATAA	TACAGTTTTT	TAGTTTTTGCT	GGCCGCATCT
8601	TCTCAAATAT	GCTTCCCAGC	CTGCTTTTCT	GTAACGTTCA	CCCTCTACCT
8651	TAGCATCCCT	TCCCTTTGCA	AATAGTCCTC	TTCCAACAAT	AATAATGTCA
8701	GATCCTGTAG	AGACCACATC	ATCCACGGTT	CTATACTGTT	GACCCAATGC
8751	GTCTCCCTTG	TCATCTAAAC	CCACACCGGG	TGTCATAATC	AACCAATCGT
8801	AACCTTCATC	TCTTCCACCC	ATGTCTCTTT	GAGCAATAAA	GCCGATAACA
8851	AAATCTTTGT	CGCTCTTCGC	AATGTCAACA	GTACCCTTAG	TATATTCTCC
8901	AGTAGCTAGG	GAGCCCTTGC	ATGACAATTC	TGCTAACATC	AAAAGGCCTC
8951	TAGGTTCCCT	TGTTACTTCT	TCCGCCGCCT	GCTTCAAACC	GCTAACAATA
9001	CTGGGGCCCA	CCACACCGTG	TGCATTGCGT	ATGTCTGCCC	ATTCTGCTAT
9051	TCTGTATACA	CCCGCAGAGT	ACTGCAATTT	GACTGTATTA	CCAATGTCAG
9101	CAAATTTTCT	GTCTTCGAAG	AGTAAAAAAT	TGTACTTGGC	GGATAATGCC
9151	TTTAGCGGCT	TAAGTGTGCC	CTCCATGGAA	AAATCAGTCA	AGATATCCAC
9201	ATGTGTTTTT	AGTAAACAAA	TTTTGGGACC	TAATGCTTCA	ACTAACTCCA
9251	GTAATTCCTT	GGTGGTACGA	ACATCCAATG	AAGCACACAA	GTTTGTTTGC
9301	TTTTTCGTGCA	TGATATTAAA	TAGCTTGGCA	GCAACAGGAC	TAGGATGAGT
9351	AGCAGCACGT	TCCTTATATG	TAGCTTTCGA	CATGATTTAT	CTTCGTTTCC
9401	TGCAGGTTTT	TGTTCTGTGC	AGTTGGGTTA	AGAATACTGG	GCAATTTTCA
9451	GTTTTTCTAA	CACCACATAT	CGGTATATAT	ACCAATCTAA	GTCTGTGCTC
9501	CTTCCTTCGT	TCTTCCTTCT	GCTCGGAGAT	TACCGAATCA	AAAAAATTTT
9551	AAAGAAACCG	GAATCAAAAA	AAAGAACAAA	AAAAAAAAAAG	ATGAATTGAA
9601	ACCCCCCCCC	CCCCCGATGC	GCCGCGTGCG	GCTGCTGGAG	ATGGCGGACG
9651	CGATGGATAT	GTTCTGCCAA	GGGTTGGTTT	GCGCATTAC	AGTTCCTCCG
9701	AAGAATTGAT	TGGCTCCAAT	TCTTGGAGTG	GTGAATCCGT	TAGCGAGGTG
9751	CCGCCGGCTT	CCATTACAGT	CGAGGTGGCC	CGGCTCCATG	CACCGCGACG
9801	CAACGCGGGG	AGGCAGACAA	GGTATAGGGC	GGCGCCTACA	ATCCATGCCA
9851	ACCCGTTCCA	TGTGCTCGCC	GAGGCGGCAT	AAATCGCCGT	GACGATCAGC
9901	GGTCCAGTGA	TCGAAGTTAG	GCTGGTAAGA	GCCGCGAGCG	ATCCTTGAAG
9951	CTGTCCCTGA	TGGTCGTCAT	CTACCTGCCT	GGACAGCATG	GCCTGCAACG
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10051	ATCCAGCCTC	GCGTCGCGAA	CGCCAGCAAG	ACGTAGCCCA	GCGCGTCGGC
10101	CGCCATGCCG	GCGATAATGG	CCTGCTTCTC	GCCGAAACGT	TTGGTGGCGG
10151	GACCACTGAC	GAAGGCTTGA	GCGAGGGCGT	GCAAGATTCC	GAATACCGCA
10201	AGCGACAGGC	CGATCATCGT	CGCGCTCCAG	CGAAAGCGGT	CCTCGCCGAA
10251	AATGACCCAG	AGCGCTGCCG	GCACCTGTCC	TACGAGTTGC	ATGATAAAGA
10301	AGACAGTCAT	AAGTGCGGCG	ACGATAGTCA	TGCCCCGCGC	CCACCGGAAG
10351	GAGCTGACTG	GGTTGAAGGC	TCTCAAGGGC	ATCGGTGCGC	GCTCTCCCTT
10401	ATGCGACTCC	TGCATTAGGA	AGCAGCCCAG	TAGTAGGTTG	AGGCCGTTGA
10451	GCACCGCCGC	CGCAAGGAAT	GGTGCAATG	AGGAGATGGC	GCCCAACAGT
10501	CCCCCGGCCA	CGGGCCGTGC	CACCATAACC	ACGCCGAAAC	AAGCGCTCAT
10551	GAGCCCGAAG	TGGCGAGCCC	GATCTTCCCC	ATCGGTGATG	TCGGCGATAT
10601	AGGCGCCAGC	AACCGCACCT	GTGGCGCCGG	TGATGCCGGC	CACGATGCGT
10651	CCGGCGTAGA	G			





**FIG. 18****Cytotoxicity Profile:****Genotoxicity Profile:**



**FIG. 19****Cytotoxicity Profile:****Genotoxicity Profile:**



## GREEN SCREEN RESULTS

TEST COMPOUND	GenC01 Strain			Test Range		GenT01 Strain	Test Range		FP	Carcinogenicity	Ames Test	ML Test	UmuC Test	MNT In vitro	MNT In vivo	Chrom Abs
	CAS No.	Cytotoxicity	LEC / $\mu\text{g ml}^{-1}$	$\mu\text{g ml}^{-1}$	mM		Genotoxicity	LEC / $\mu\text{g ml}^{-1}$								
2-Acetylaminofluorene	53-96-3	++	28.8	115	0.52	-	-	115	0.52	+	+/- (MA)	+	+	+	-	+
Acetylsalicylic Acid	50-76-2	-		867	4.81	-	-	867	4.81		-		-	-	+/-	+
Actinomycin D	50-76-0	+	62.5	500	0.40	-	-	500	0.40	+	-	+	-			+
Acycloguanosine	59277-89-3	+	56.3	112.5	0.50	-	-	112.5	0.50	-						
9-Aminoacridine	90-45-9	++	8	128	0.55	-	-	128	0.55				+			+(p)
2-Aminoanthracene	613-13-8	++	14.25	57	0.29	-	-	57	0.29	+	+	+	+	+		+
2-Amino-4-nitrophenol	99-57-0	++	19.25	77	0.50	++	++	77	0.50	+/-	+/- (MA)	+		-		+
4-Aminophenol	123-30-8	-		504	4.62	-	-	504	4.62		-	+		+		
3-Amino-1,2,4-Triazole	61-82-5	+	840	840	9.99	+	+	840	9.99	+/-	-	-				-
Ampicillin (Na salt)	69-52-3	-		8000	22.9	-	-	8000	22.9	-	-	-			-	
Aniline	62-53-3	++	2553	20420	219.3	-	-	20420	219.3	+/-	-	+		-	+	+
o-Anisidine	90-04-0	++	14.2	437	3.55	-	-	437	3.55	+	+/- (MA)	+	-			+
Aphidicolin	36966-21-1	+	20	20	0.06	+	+	20	0.06		-	+	-			
AraC	147-94-4	-		1400	5.76	-	-	1400	5.76	-	-	+		+	+	+
5-Azacytidine	320-67-2	++	625	1250	5.12	++	++	625	5.12	+/-	+	+		+	+	
AZT	30516-87-1	+	3675	7350	27.5	-	-	7350	27.5	+/-	-	+		+	+	+
Benzaldehyde	100-52-7	++	2625	5220	49.2	+	+	1313	49.2	+/-	-	+				+
Benzo(a)pyrene	50-32-8	-		26	0.10	+	+	26	0.10	+	+/- (MA)	+	+	+	+/-	+
Benzoyl Chloride	98-98-4	++	1515	12120	86.22	+	+	304	86.22		+		+	+	+	+
Bleomycin Sulfate	9041-93-4	++	5	5	0.0035	++	++	2.5	0.0035	+	+/-	+	+	+	+	+
Cadmium Chloride	10108-64-2	+	2	2	0.011	-	-		0.011	+/-	-	-	-	+/-	+/-	+
Caffeine	58-08-2	++	266	531	2.73	-	-	531	2.73	-	-	+	-	+/-	+/-	+
Catechol	120-80-9	++	177	880	7.99	++	++	599	7.99	+/-	-	+	-	-	-	
Celastrol	64485-93-4	++	238	238	0.50	++	++	238	0.50							
Chlorambucil	305-03-3	+	125	250	0.82	-	-	250	0.82	+	+/-			+	+	+
Chloramphenicol	56-75-7	+	162	162	0.50	++	++	10.1	0.50	-	-	+	-	-	-	
Chromomycin A3	7059-24-7	-		10	0.0085	-	-		0.0085		-		-			
Cimetidine	51481-61-9	+	125	125	0.50	++	++	31.3	0.50	-						
Cisplatin (without DMSO)	15663-27-1	++	2.34	150	0.50	+	+	18.75	0.12	+	+/-		+			
Colchicine	64-86-8	-		853	2.14	+	+	427	2.14	+	-	-		+	+/-	
Crotonaldehyde	4170-30-3	++	26.5	1692	24.1	++	++	85.2	24.1	+	+	+		+	+	
Cumene Hydroperoxide	80-15-9	++	65.4	519	3.41	-	-	104	0.88		+	+	+			
Cycloheximide	66-81-9	++	0.03	1	0.0036	-	-	1450	5.15		-	+		+		
Daunorubicin	23541-50-6	++	0.5	250	0.44	++	++	0.25	0.0009	+	+/-		+			
3,5-Dichlorophenol	591-35-6	++	12.5	25	0.15	-	-	25	0.1534		-					
Dicumyl Peroxide	80-43-3	++	0.3	0.3	0.0011	-	-	0.3	0.0011		-					
Dieldrin	60-57-1	-	19.8	39.6	0.10	-	-	39.6	0.10	+/-	-	+	-	+	+	+
Diethylnitro-4-methylcoumarin	91-44-1	++	59	118	0.51	-	-	118	0.51		-					
1,2-Dimethylhydrazine HCl	306-37-6	++	330	1330	10.00	++	++	665	10.00	+	-			+		
Econazole Nitrate	24169-02-6	++	25	50	0.11	++	++	2.5	0.011		-					
Ellipticine	519-23-3	++	4.39	562	2.28	++	++	12.5	2.280		+/-	+				
1,2-Epoxybutane	106-98-7	+	8370	16740	232	+	+	16740	232	+/-	+/-	+	+		-	
Ethidium Bromide	1239-45-8	-		12.5	0.032	++	++	6.25	0.016		+/- (MA)	+	+	+	+	+
7-Ethoxycoumarin	31005-02-4	-		129	0.68	++	++	32.3	0.68							
Ethyl Acrylate	140-88-5	++	4605	18420	184.0	+	-		183.98	+/-	-	+				+
Ethylenechloride	107-15-3	-	224.8	450	7.49	-	-	450	7.49	-	-					



Fig.20 [2/3]

Ethyl methanesulfonate	62-50-0	-		100	0.81	++	25	100	0.81		+	+/-	+																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																												</
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Fig.20 [3/3]

Thiourea	62-56-6	++	10050	20100	284	++	1256	5000	65.7		+	-	+/-		
Titanium Dioxide	13463-67-7	-		179	2.24	-		179	2.24		+/-	-	-	+	
Trichloroacetonitrile	545-06-2	++	3.6	28.8	0.20	++	1.8	28.8	0.20			+/-		-	
Trifolyl Phosphate	1330-78-5	-		572	1.55	++	143	572	1.55		-	-			
Urethane	51-79-6	++	10300	20800	231	++	10300	10300	115.60		+	-	+	+	+
Vanillin	121-33-5	++	21.25	170	1.12	-		152	1.00		-	-	-	-	-
Vinblastine	143-67-9	++	62.5	250	0.28	-		250	0.28		-			+	+

## KEY

- Negative  
 + Positive  
 ++ Strong Positive  
 +/- Results vary between reports  
 MA Metabolic activation required to obtain positive result.  
 LEC Lowest effective concentration  
 FP Fluorescence polarisation used to reveal the result.  
 (p) Polyploid problems increase significantly



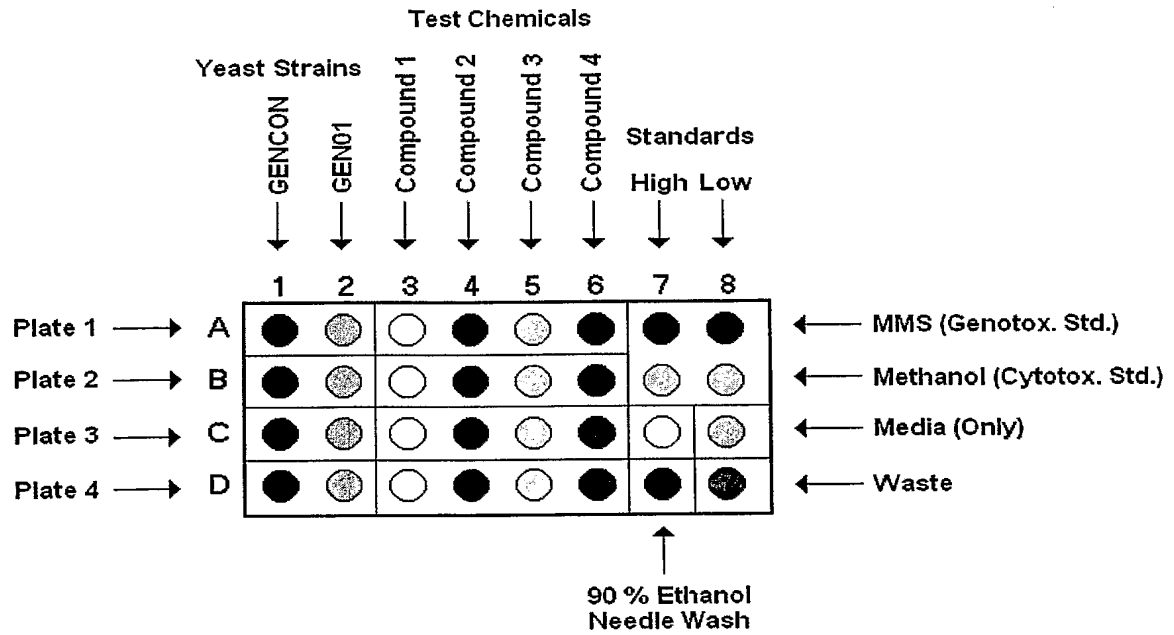
FIG.21

TEST COMPOUND	GSA	Ames	S9	TEST COMPOUND	GSA	Ames	S9
2-Amino-4-nitrophenol	++	+	+	2-Acetamidofluorene	-	+	+
Ethidium Bromide	++	+	+	2-Aminoanthracene	-	+	+
Neutral Red	++	+	+	o-Anisidine	-	+	+
Proflavin Hemisulfate	++	+	+	8-Hydroxyquinoline	-	+	+
5-Azacytidine	++	+	-	Isobutyl Nitrite	-	+	+
Bleomycin Sulfate	++	+	-	N-Nitrosodiphenylamine	-	+	+
Crotonaldehyde	++	+	-	9-Aminoacridine	-	+	-
Daunorubicin	++	+	-	Chlorambucil	-	+	-
Ellipticine	++	+	-	Cumene Hydroperoxide	-	+	-
Ethyl methanesulfonate	++	+	-	Hydroquinone	-	+	-
Furazolidone	++	+	-	ICR191 Acridine Mutagen	-	+	-
Hydrazine monohydrate	++	+	-	Nitrofurantoin	-	+	-
Hydroxyurea	++	+	-	N-Nitrosodimethylamine	-	+	-
Methyl methanesulfonate	++	+	-	4,4-Oxydianiline	-	+	-
MNNG	++	+	-	Quercetin	-	+	-
Nalidixic Acid	++	+	-	Sodium Selenite	-	+	-
4-Nitroquinoline-N-oxide	++	+	-	Acetylsalicylic Acid	-	-	
N-Nitroso-N-ethyl urea	++	+	-	Actinomycin D	-	-	
N-Nitroso-N-methyl urea	++	+	-	4-Aminophenol	-	-	
Sodium Azide	++	+	-	Ampicillin (Na salt)	-	-	
Streptonigrin	++	+	-	Aniline	-	-	
Trichloroacetonitrile	++	+	-	AraC	-	-	
Benzo(a)pyrene	+	+	+	AZT	-	-	
1-Naphthylamine	+	+	+	Cadmium Chloride	-	-	
Benzoyl Chloride	+	+	-	Caffeine	-	-	
Cisplatin (without DMSO)	+	+	-	Chromomycin A3	-	-	
1,2-Epoxybutane	+	+	-	Cycloheximide	-	-	
Hexamethylenetetramine	+	+	-	3,5-Dichlorophenol	-	-	
Hydrogen Peroxide	+	+	-	Dicumyl Peroxide	-	-	
Mechlorethamine HCl	+	+	-	Dieldrin	-	-	
Mitomycin C	+	+	-	Diethylamino-4-methylcoumarin	-	-	
3-Amino-1,2,4-triazole	+	-		Ethyl Acrylate	-	-	
Aphidicolin	+	-		Ethylenediamine	-	-	
Benzaldehyde	+	-		Methyl Carbamate	-	-	
Colchicine	+	-		Methyl Methacrylate	-	-	
Etoposide	+	-		Nicotine	-	-	
Methyl viologen	+	-		Nitrobenzene	-	-	
Psoralen	+	-		Phenol	-	-	
Catechol	++	-		Sulfisoxazole	-	-	
Chloramphenicol	++	-		Taxol	-	-	
1,2-Dimethylhydrazine HCl	++	-		Tetracycline HCl	-	-	
Econazole Nitrate	++	-		Titanium Dioxide	-	-	
Methapyrilene HCl	++	-		Vanillin	-	-	
Phthalic acid bis(2-ethylhexyl) ester	++	-		Vinblastine	-	-	
Safrole	++	-		S9 column records requirement for Ames result  + S9 required - S9 not required			
Sulfamethoxazole	++	-					
Thiourea	++	-					
Tritolyl Phosphate	++	-					
Urethane	++	-					

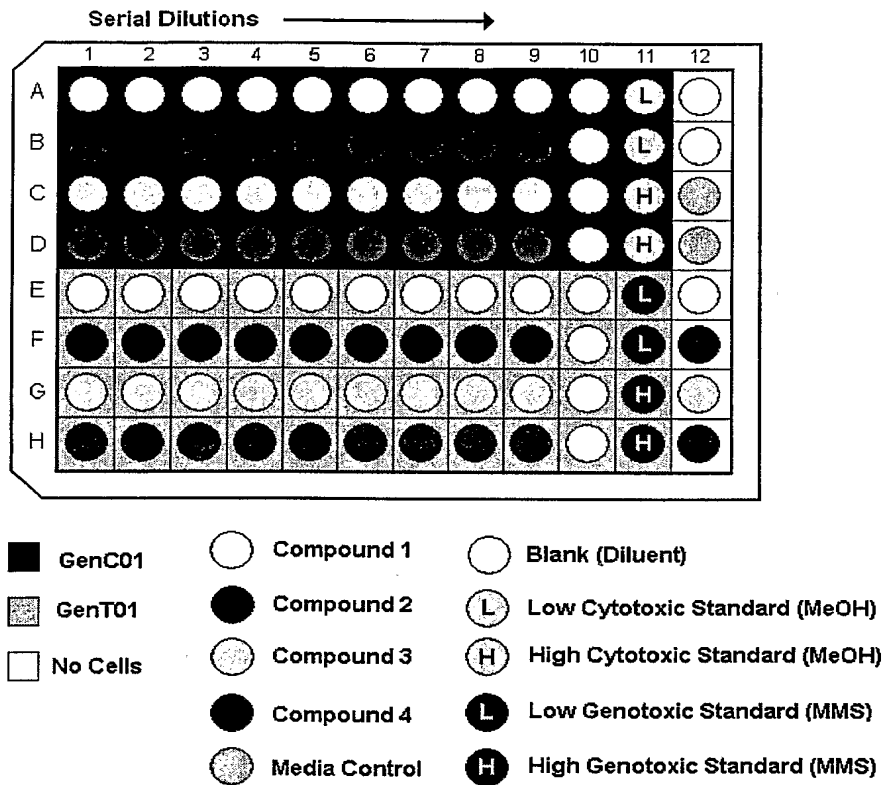
Table 3

Table 3



**FIG. 22****Greenrack loading sequence**



**FIG. 23****Microplate layout**

PCT/GB2004/003337

